Advances in Experimental Medicine and Biology 744

William S. Spielman
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# RAMPs

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# RAMPs

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RAMPs
William S. Spielman and Narayanan Parameswaran

# RAMPs

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Printed in the USA.

Springer Science+Business Media, LLC, 233 Spring Street, New York, New York 10013, USA
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The chapters in this book are available in the Madame Curie Bioscience Database.
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*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran. Landes Bioscience / Springer Science+Business Media, LLC dual imprint / Springer series: Advances in Experimental Medicine and Biology.

ISBN: 978-1-4614-2363-8



**Library of Congress Cataloging-in-Publication Data**

RAMPs / edited by William S. Spielman, Narayanan Parameswaran.
p. ; cm. -- (Advances in experimental medicine and biology ; 744)
Includes bibliographical references and index.
ISBN 978-1-4614-2363-8
I. Spielman, William S., 1947- II. Parameswaran, Narayanan, 1970- III. Series: Advances in experimental medicine and biology ; 744. 0065-2598
[DNLM: 1. Receptor Activity-Modifying Proteins. W1 AD559 v.744 2012 / QU 55.7]
LC classification not assigned
572'.65--dc23

2011046154

# PREFACE

G-protein coupled receptors (GPCRs) comprise one of the largest families of receptors studied by receptor biologists across the globe. It is also the largest family of receptors that is targeted by the pharmaceutical industry for treating various disease conditions. In this respect, questioning how GPCRs function is one of the most critical questions not only in the endeavor of drug development but also in understanding the basic physiological processes. Among the many GPCRs discovered, the calcitonin family of receptors comprise of members that regulate a number of physiological processes and are involved in many pathological conditions. Therefore, understanding how these receptors function is a critical question in the field. When Foord and his colleagues discovered that a single transmembrane protein called Receptor Activity Modifying Proteins (RAMPs) could modulate the surface expression of GPCRs of the calcitonin family, it widely opened the field of receptor life cycle. Hundreds of studies have confirmed the importance of RAMPs in the life cycle of this receptor family. This volume is intended to be a source of these basic studies that range from biochemistry to molecular biology to receptor pharmacology and physiology. The authors who have contributed to the chapters in the book are well known in the RAMPs field. This book is intended to be a source of information for scientists at different stages of their career including graduate students, post-doctoral fellows and senior scientists who are new to the RAMPs field. The chapters are also organized in a way that makes it easy to navigate through the book.

The first chapter provides a general introduction to RAMPs including its discovery. The second chapter (by Poyner and colleagues) and the third chapter (by Bomberger and colleagues) are intended to provide in depth discussion on how the different RAMP isoforms can regulate various aspects of receptor signaling especially stimulated with calcitonin gene related peptide (CGRP) and adrenomedullin. In addition, in the third chapter, Bomberger et al discuss the differences in RAMP2 and -3 with regard to their role in receptor trafficking.

Initial discovery of RAMPs suggested that they regulate GPCRs of the Class II family. Subsequent research by Dr. Henley's group demonstrated a role for RAMPs in the surface expression of Class III GPCRs. Dr. Henley and his colleagues in Chapter 4 discuss this novel feature of RAMPs.

A crucial aspect to the advancement of the RAMP field has been the generation of RAMP transgenic/knockout mice. These RAMP transgenic/knockout mice have greatly facilitated our understanding of the physiological role of these proteins. Dr. Caron's group, which pioneered many of these experiments, discusses the studies in Chapter 5. From a therapeutic point of view, it is important to consider whether RAMPs are potential drug targets. Sexton and colleagues discuss in Chapter 6, the usefulness of RAMPs as drug targets.

Subsequent to the discovery of RAMPs, scientists working on other receptors realized there could be proteins similar to RAMPs regulating their receptor of interest. In one particular case, Dr. Matsunami's group discovered RAMP-like proteins that regulate odorant receptors. Dr. Matsunami and his colleague discuss, in Chapter 7, this novel role of RAMP-like proteins in the regulation of odorant receptors.

RAMP expression is modulated in various diseases and Dr. Wang and his group discuss this in Chapter 8. Finally, in Chapter 9, we discuss some of the recent high impact studies that will help provide a perspective on the role of RAMPs in diseases such as cancer, hypertension and asthma.

We thank all the authors for their insightful and comprehensive contributions to this work. We also thank the publisher for their patience and suggestions in bringing *RAMPs* together in a timely manner. Editing this volume has been a rewarding experience for us, especially in terms of learning further about the functions of these single transmembrane proteins. Receptor biology is a rapidly expanding field and with the advances in cell and molecular biology and in vivo techniques, it is very likely that the field of RAMPs will explode further and many unanswered questions will be answered with in the next few years. We hope this book will serve as a useful resource for those scientists interested in not only the field of RAMPs but also in the general field of GPCR biology.

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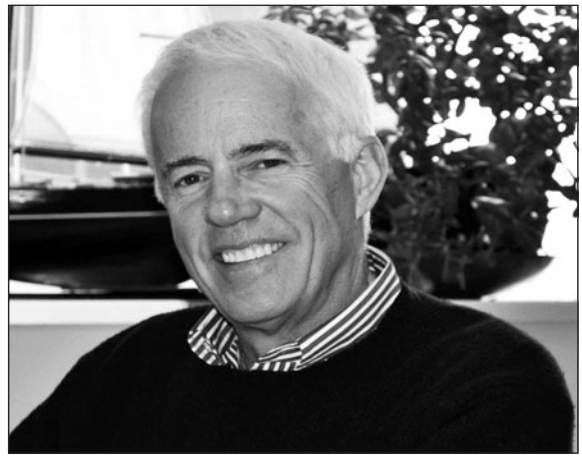

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# CONTENTS

CHAPTER 1

# INTRODUCTION TO RAMPs

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**Abstract:** Receptor activity modifying proteins (RAMPs) are single transmembrane proteins discovered for their role in the regulation of translocation of certain G-protein coupled receptors (GPCRs) to the plasma membrane. Since its discovery in 1998, several pivotal advances have been made in understanding the function of this family of proteins. This chapter provides a basic introduction to RAMPs as well as details on the various chapters in this book.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

In 1998, Foord and his colleagues ended years of frustration among adrenomedullin (AM) and calcitonin gene related peptide (CGRP) receptor biologists by discovering a single transmembrane accessory protein called receptor-activity modifying proteins.[1] Using an expression cloning strategy in Xenopus Oocytes, a 148-amino acid RAMP1 was identified initially and this protein was shown to regulate proper translocation of calcitonin receptor-like receptor (CLR) to the plasma membrane. In addition, this protein was shown to regulate CGRP responsiveness of this receptor. Data base search revealed two additional and related RAMPs, RAMP2 and RAMP3 (Fig. 1). It was then determined that RAMP1 and CLR combination gives rise to a CGRP responsive receptor whereas RAMP2 or RAMP3 and CLR combinations give rise to an adrenomedullin receptor. Prior to McLatchie et al's[1] discovery of RAMPs, adrenomedullin/CGRP receptor biologists were frustrated because heterologous expression of CLR alone gave rise to a CGRP receptor only in specific cell types.[2,3] McLatchie et al's report clarified that the presence of a particular RAMP in a specific cell line dictated whether CLR expression would give rise to a CGRP receptor or an adrenomedullin receptor, thus ending the confusion.

Subsequent studies have found that of the two peptides, amylin and calcitonin (both of which signal via Calcitonin receptor (CTR)), amylin's interaction with CTR is dependent on RAMPs, while that of calcitonin is not (For a review, see ref. 4). Taken together, this unique diversity in receptor interaction (four ligands, three RAMPs, two GPCRs) provided a paradigm shift in our understanding of GPCR biochemistry (Fig. 2). Recent findings suggest that RAMPs may diversify ligand-receptor interaction to a much larger scale within the Class II GPCRs. In this regard, Christoupoulos et al[5] showed that, VPAC1 receptor (vasoactive intestinal polypeptide/ pituitary adenylate cyclase-activating peptide receptor) interacts with all three RAMPs, whereas the glucagon and PTH1 (parathyroid hormone receptor) interact only with RAMP2. Interestingly PTH2 receptor was shown to interact with RAMP3.[5] In a recently published study, Harikumar et al[6] present further evidence that another member of the Class II GPCR, namely the secretin receptor associates with RAMP3, but not RAMP1 or RAMP2. Together these studies not only expand the repertoire of GPCRs that RAMPs interact with, they also provide evidence for selectivity of GPCRs for certain RAMP members. In addition to interaction with members of Class II GPCRs, previous studies from Dr. Henley's group have shown that Calcium Sensing receptor (CaSR), which belongs to the Class III GPCR, is also regulated by RAMPs.[7] The role of RAMPs in regulating a variety of GPCRs is only expanding and this book provides a detailed account of

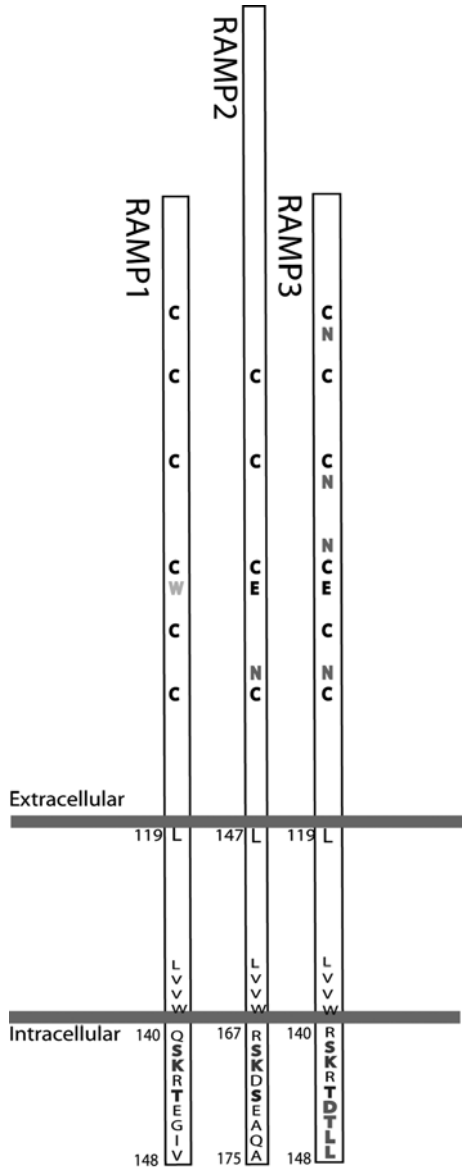


**Figure 1.** Structural comparison of RAMP1, 2 and 3: RAMPs share ~30% sequence identity. RAMP2 is the least conserved, being 26 amino acids longer than RAMP1 and RAMP3.[1] The conserved cysteine residues (C) are in the extracellular domain of all three RAMPs; the N-terminal glycosylation sites (N) are also shown. The tryptophan (W) in the extracellular domain of RAMP1 is crucial for the species selectivity of the CGRP1 receptor antagonist (BIBN44096BS). Conserved amino acids in the transmembrane domain are shown. The three RAMPs contain a conserved Ser-Lys (S, K; brown) sequence in the intracellular domains. Putative phosphorylation site (threonine in RAMP1 and 3; serine in RAMP2) are shown in blue; the PDZ-binding motif (Asp-Thr-Leu-Leu) in RAMP3 is shown in green. Reprinted from Parameswaran N, Spielman WS. Trends Biochem Sci 2006; 31(11):631-638;[4] with permission from Elsevier. A color version of this figure is available at www.landesbioscience.com/curie.

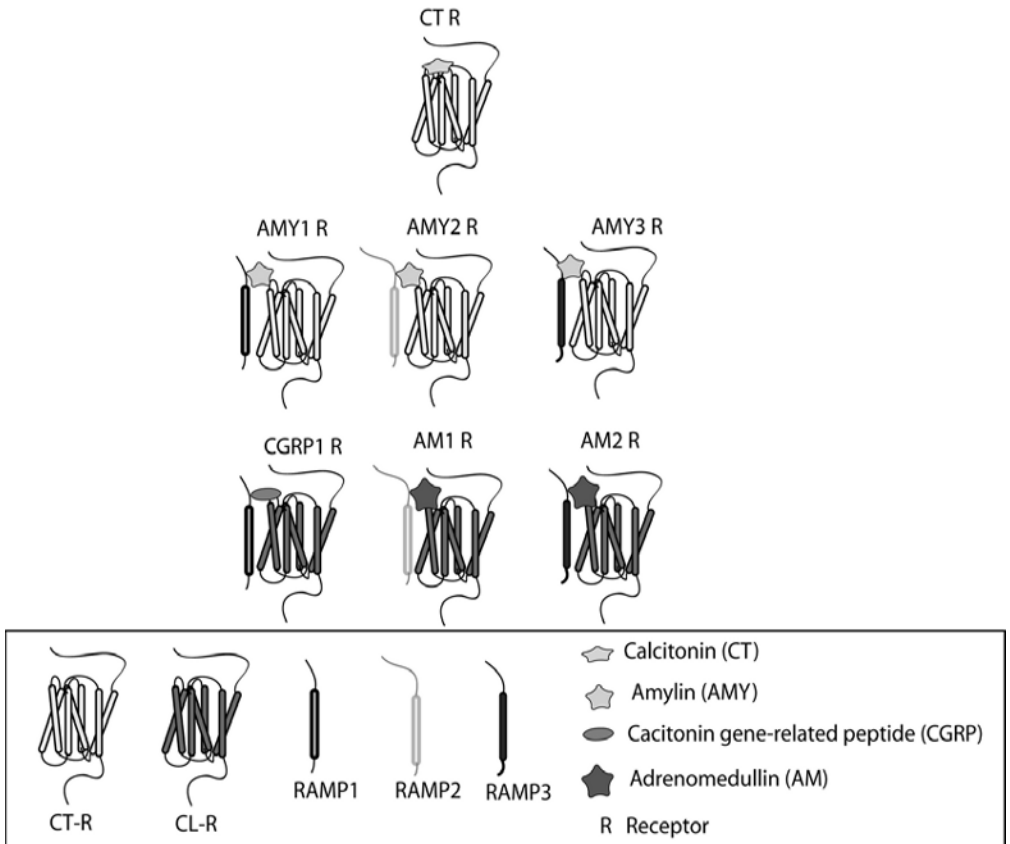


**Figure 2.** Binding of the calcitonin family of peptides to CLR and CTR: regulation by RAMPs. A) The binding of calcitonin to its receptor, CTR, does not require RAMPs. B) Interaction of CTR with RAMP1 (i), RAMP2 (iii) and RAMP3 generates AMY1, AMY2 and AMY3 receptors, respectively. C) Interaction of CLR with RAMP1 generates a CGRP1 receptor (i), whereas its interaction with RAMP2 (ii) and RAMP3 (iii) yields AM1 and AM2 receptors, respectively. Reprinted from Parameswaran N, Spielman WS. Trends Biochem Sci 2006; 31(11):631-638;[4] with permission from Elsevier.

what we know so far in terms of the different biochemical and physiological functions of RAMPs with regard to the life-cycle of their cognate GPCRs that they regulate.

## REGULATION OF CGRP RESPONSIVENESS BY RAMP1

Since the discovery of RAMPs in the regulation of CGRP responsiveness and signaling, a number of studies have been initiated to understand the biochemical and structural aspects of CLR interaction with RAMP1, as well as the interactions of CGRP with CLR and RAMP1.[8-22] These studies have also shed light into the binding of nonpeptide antagonists of CGRP receptor.[23,24] In Chapter 2 of this book, Poyner and his colleagues expand on these findings and discuss in detail the biochemical and functional ramifications of CGRP interaction with the RAMP1-CLR complex.

## REGULATION OF CLR LIFE-CYCLE BY RAMPs

Upon activation by ligands, GPCRs undergo a number of life-cycle events including G-protein-dependent and independent signaling, receptor endocytosis, degradation or recycling.[25-27] Because CLRs belong to the GPCR family, a number of studies have examined these life-cycle events, especially in the context of how RAMPs might modulate these processes.[28-33] Previous studies have shown that these specific life-cycle processes of CLR can be regulated in a RAMP-specific manner.[30,31] In this regard, RAMP3 has been shown to regulate internalization of CLR as well as post-endocytic receptor trafficking in response to adrenomedullin. Interestingly, these roles of RAMPs are restricted to RAMP3 because of their PDZ domain at the c-terminus. Although RAMP2-CLR and RAMP3-CLR are adrenomedullin receptors, only RAMP3 regulates these important life-cycle events in CLR biology. It remains to be seen however, whether these differences in RAMPs in regulating CLR life-cycle has important physiological consequences in vivo. In Chapter 3, Bomberger et al, discuss these aspects of RAMPs in detail.

## RAMPs AND CALCIUM SENSING RECEPTOR

Even though RAMPs were originally discovered to regulate members of the Class II GPCRs, Dr. Henley's group first provided evidence that RAMPs can also regulate Class III GPCRs.[7] In this regard, they showed that cell surface expression of CaSR (a Class III GPCR) is regulated by RAMP1 or RAMP3, but not RAMP2. For these experiments, these scientists used a novel CaSR

fused to the pH sensitive variant of GFP called Super Ecliptic pHluorin, to understand the regulation of trafficking of this receptor by RAMPs. In Chapter 4, Bouschet et al describe these experiments and their findings in detail.

## ANIMALS MODELS AND RAMPs

Mechanistic understanding of receptor biology involves a number of biochemical approaches. However physiological roles of the receptors and ligands depend heavily on genetically modified animal models. Since its initial discovery, mice with targeted deletion of RAMP1 or 2 or 3 have been generated and their phenotype examined.[34-37] Interestingly, homozygous deletion of RAMP2, but not RAMP1 or RAMP3 is lethal, thus suggesting important role for RAMP2 in development that cannot be compensated by other RAMPs. Transgenic models over-expressing RAMP1 or RAMP2 have also been described.[38,39] In Chapter 5, Caron and her colleagues take a detailed look at the available animal models for RAMPs and comprehensively describe the phenotypes of these available models.

## RAMPs AS DRUG TARGETS

It is well known that more than 50% of the pharmacological drugs in the market are directed towards GPCRs. RAMP1/CLR complex, a receptor for CGRP is currently being targeted for the treatment of migraine headache. Several small molecule nonpeptide antagonists have also been described for CGRP receptor. Some of these antagonists have binding sites on RAMP1. Thus, by virtue of being in complex with CLRs and because of their binding to the ligands, RAMPs are also potential drug targets for treatment of diseases where aberrant activation of these receptor complexes contribute to disease pathogenesis. Although discovered as an accessory protein to CLR and other receptors, their potential impact in drug development could be significant. As described in Chapter 6 by Sexton et al, RAMP-receptor complexes as well as RAMPs themselves can be targeted for drug development. Given the multitude of receptors that RAMPs interact with, targeting RAMPs alone could have multiple effects depending on the specific RAMP targeted. This is clearly an area that is likely to expand in the near future and will be an area that the pharmaceutical companies will likely focus on.

## RTP AND REEP

Discovery of RAMPs facilitated the idea of accessory proteins regulating proper receptor translocation and opened up the field by identification of more RAMP-like proteins. In the seventh chaper, Mainland and Matsunami describe their recent findings on a family of proteins called RTP (Receptor-Transporting protein) and REEP (Receptor Expression Enhancing Protein), which function very similar to that of RAMPs, particularly in the regulation of odorant receptors. When mammalian odorant receptors are expressed in heterologous systems, they are mostly retained in the endoplasmic reticulum. Matsunami's lab made the initial discovery that when odorant receptors are co-expressed with RTP/REEP proteins, then they are correctly targeted to the cell surface.[40] While this function of RTP/REEP proteins is quite similar to that of RAMPs, these proteins do not appear to alter the pharmacology of the odorant receptors that they complex with. As concluded in Chapter 7 by Mainland and Matsunami, it is highly critical to test whether these proteins are important in vivo and whether they play any role in developmental processes. Generation and comparative phenotyping approaches as described for the RAMPs are therefore necessary to further understand the physiological roles of RTP/REEP family of protein.

## REGULATION OF RAMPs EXPRESSION

Expression of RAMPs has been detected in many tissues in mouse, human as well as other species (see Table 1). Because over-expression, knockdown or knockout of RAMPs have functional effects on the receptor signaling, it has been postulated that changes in the expression of RAMPs may be modulated in disease conditions leading to altered signal transduction. In support of this idea, many studies have examined changes in expression of RAMPs in different disease models in animals as well as in many human diseases. In Chapter 8, Dr. Wang and his colleagues outline in detail, the modulation of RAMP expression in various disease conditions including cardiovascular, cancer, sepsis, cirrhosis, glomerulonephritis, diabetes and Parkinson's disease. While some of these observations are in human diseases, others have been observed in animal models of disease. Further studies are necessary to understand whether the changes in RAMP expression in these disease conditions alter receptor signaling to the extent that it modulates the pathogenesis of disease.

**Table 1.** RAMP1, RAMP2 and RAMP3 mRNA expression levels in mouse and human tissues assessed by northern blot analysis. The relative expression levels are based on data from reference 45 for mouse and reference 1 for human tissues. + denotes the level of expression (i.e., +++++, high expression; +, low expression); ? denotes little or no signal.

| | RAMP1 | | RAMP2 | | RAMP3 | |
|---|---|---|---|---|---|---|
| TISSUE | Mouse | Human | Mouse | Human | Mouse | Human |
| BRAIN | +++++ | ++ | ++++ | ? | +++++ | ++ |
| HEART | +++ | +++++ | +++ | +++++ | ++ | +++++ |
| LUNGS | +++++ | ? | +++++ | ++++ | + | ++++ |
| KIDNEY | ? | ? | +++ | + | +++ | +++ |
| PANCREAS | ++ | +++++ | ++ | + | ? | +++ |
| SKELETAL MUSCLE | ++++ | +++++ | ++++ | ++++ | ? | +++ |
| LIVER | +++ | + | +++ | ? | ? | + |

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While this book was in the final stages of preparation, a few high impact studies were published that further demonstrated an important role for RAMPs in various pathophysiological functions.[41-44] In Chapter 9, the final chapter of this book, we discuss these studies to provide a perspective on these functions of RAMPs in cancer biology, hypertension and asthma.

## CONCLUSION

The discovery of RAMPs has caused a significant paradigm shift in our understanding of cell surface translocation of GPCRs. The chapters compiled here provide detailed insight into this function of RAMPs on a range of topics both from biochemical and physiological perspective of GPCR regulation.

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CHAPTER 2

# RAMPs AND CGRP RECEPTORS

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**Abstract:** Receptor activity modifying protein 1 (RAMP1) forms a complex with calcitonin receptor-like receptor (CLR) to produce the receptor for calcitonin gene-related peptide (CGRP). RAMP1 has two main roles. It facilitates the cell-surface expression of CLR. It is also essential for the binding of CGRP to the receptor. It seems likely that Y66, F93, H97 and F101, amongst other residues, form a binding site for CLR. These cluster together on the same face of the extracellular portion of RAMP1, probably close to where it enters the plasma membrane. Residues at the other end of RAMP1 are most likely to be involved in CGRP recognition, although it is currently unclear how they do this. Within this area, W74 is important for the binding of the nonpeptide antagonist, BIBN4096BS, although it does not seem to be involved in the binding of CGRP itself. It has been shown that there is an epitope within residues 23-60 of CLR that are essential for RAMP recognition. Under some circumstances, changes in the expression of RAMP1 can alter the sensitivity of cells to CGRP, demonstrating that regulation of its levels may be of physiological or pathophysiological importance.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

# INTRODUCTION

## RAMPs and CGRP Receptors

Receptor activity modifying proteins (RAMPs) owe their discovery to the search for calcitonin gene-related peptide (CGRP) receptors. In the early 1990s, a new G-protein coupled receptor (GPCR) was cloned from rat and human sources.[1-3] This receptor showed around 50% sequence identity to the related calcitonin receptor and so was named calcitonin receptor-like receptor (CRLR; this is now abbreviated to CLR).[4] However, when expressed in most cells it was not activated by any known ligand, except for one clone of HEK293 cells where it responded to CGRP.[5] It was hypothesised that these cells expressed some kind of cofactor that allowed CLR to bind CGRP. This was proven to be the case in 1998 when RAMP1 was cloned and shown to interact with CLR to give a CGRP receptor.[6] In the same study, RAMPs 2 and 3 were shown to produce receptors for adrenomedullin (AM); these receptors are now known as AM1 and AM2 receptors.[4] The AM2 receptor shows significant affinity for CGRP.[6] Similarly, CGRP receptors also bind AM with a reasonably high affinity. Subsequently the RAMPs were also shown to interact with calcitonin receptors to give receptors for amylin,[7,8] known as the AMY1, AMY2 and AMY3 receptors. Of these, the AMY1 and AMY3 also show significant ability to interact with CGRP.[8] They will not be considered in this chapter.

Initially, the complex between the CLR and RAMP1 was known as the $CGRP_1$ receptor, reflecting concerns about possible CGRP receptor heterogeneity.[4] Subsequently this issue has been resolved so that the complex is now simply known as the CGRP receptor.[9]

## CLR and CGRP

CLR is an example of a family B GPCR; these have large N-termini, characterised by three sets of conserved disulphide bonds. The N-termini of these receptors bind the C-termini of their cognate ligands. The N-termini of the ligands interact with the transmembrane domains of the receptors and it is this interaction which activates the receptor.[10] CGRP is a 37 amino acid peptide; $CGRP_{8-37}$ is an antagonist and these are the residues which are most likely to interact with the N-terminus of CLR when it is complexed to RAMP1. There is no structure available for the N-terminus of CLR. However, it is unlikely to show much departure from those defined for a number of allied GPCRs.[11-14] There seems to be a common pattern with alpha-helices at the extreme N- and C-termini of the extracellular domain, with two or three short beta-sheets between them.

## RAMP1 AND CGRP RECEPTOR PHARMACOLOGY

### Complex Formation between CLR and RAMP1

CLR and RAMP1 appear to associate shortly after synthesis in the endoplasmic reticulum; RAMPs that do not complex with GPCRs such as CLR form homodimers.[6,15] The RAMP1:CLR complex appears to be stable both at the cell surface and during receptor internalisation.[16,17] It is possible to change from CGRP to AM receptors by overexpressing the relevant RAMP, but there is little understanding of the mechanisms that regulate this in-vivo.[7,18]

The stoichiometry of the RAMP1:CLR complex is unclear. Cross-linking studies with bis(sulfosuccinimidyl)suberate suggested that there was a 1:1 complex between RAMP1 and CLR.[17] A similar conclusion can be drawn from cross-linking experiments with [$^{125}$I]-CGRP, although the details of the labelling patterns depend on the cell line used.[15,19] However, a recent study using bimolecular fluorescence complementation with bioluminescence resonance energy transfer produced evidence for a complex with a stoichiometry of 2 CLR: 1 RAMP1.[20] The reason for the discrepancy between these studies is not clear.

### RAMP1, CLR and CGRP Binding

RAMP1 produces its effects on CLR as a result of direct protein-protein interactions.[15] Studies with chimeric receptors have established that the N-terminus of RAMP1 is the major determinant of pharmacology.[21] Subsequent work has helped delineate some of the regions within this domain that have specific roles (Figs. 1 and 2). Removal of residues 51-5 or 83-90 impaired HA-tagged RAMP1 translocation to the cell surface but did not greatly impair the ability of CGRP to cause a stimulation of cAMP. Thus the epitopes defined by these residues, whilst having some role in the formation of a functional receptor, can be considered as being of a secondary nature. Deletions between residues 41-50, 59-71 and 91-103 impaired both translocation of HA-tagged RAMP1 to the cell surface and CGRP responsiveness. However, some RAMP synthesis was taking place, as the HA tag could be detected inside the cells by ELISA. Thus epitopes within these regions are of prime importance for the association of RAMP1 with CLR and perhaps the formation of a CGRP binding site. Deletion of residues 28-33, 78-80 and 88-90 gave receptors that responded normally to CGRP but which could not be activated by AM.[22] The interpretation of deletions is not always simple, as removal of amino acids may cause significant perturbations to the folding of a protein at sites far removed from the actual deletion. However, it is interesting that when the epitopes identified in the above study are mapped onto the recent crystal structure for RAMP1,[23] some patterns can be seen (Figs. 1 and 2). The

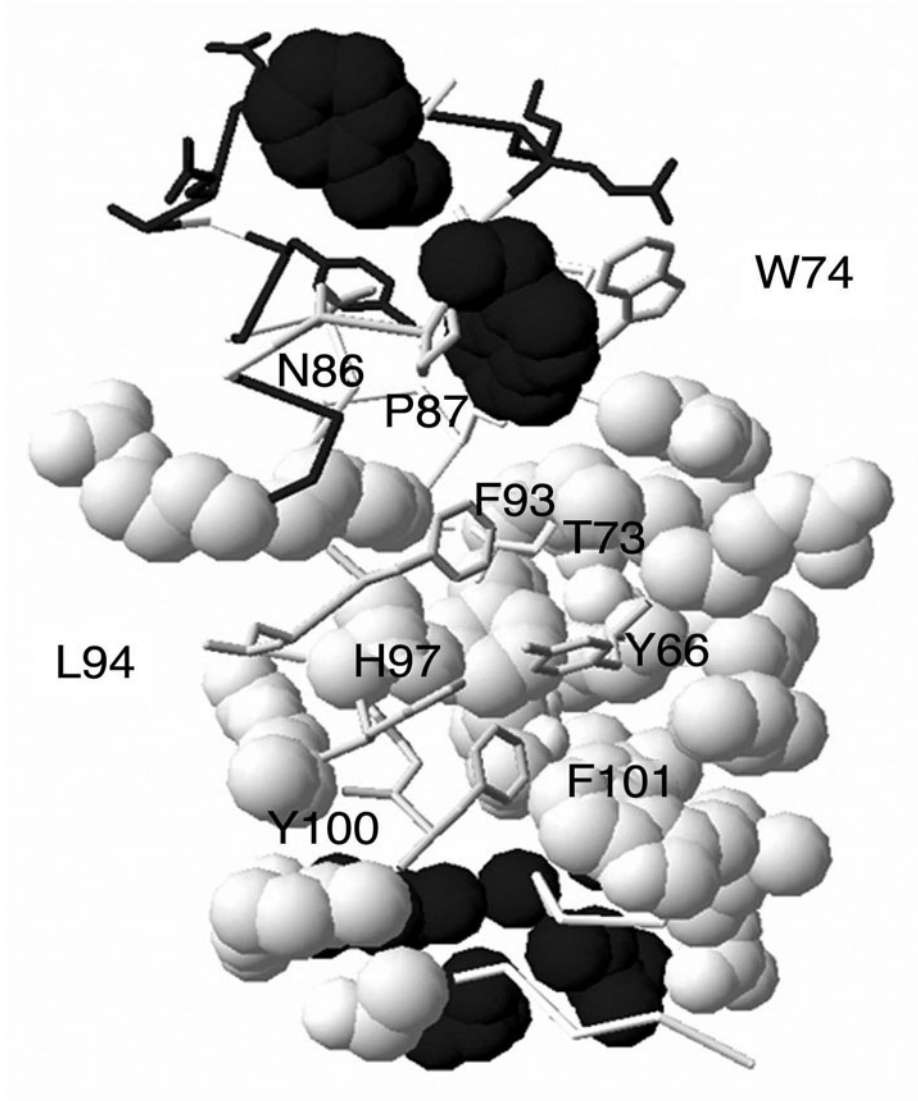


**Figure 1.** Structure of RAMP1, side view. Space filling residues shown in grey are in regions where deletions caused a major loss of CGRP- and AM-responsiveness as well as RAMP1 cell-surface expression. Space filling residues shown in black are in regions where deletions caused a major loss of RAMP1 cell-surface expression alone. Residues in line-drawn form in black are in regions where deletions altered AM responsiveness alone. Other line-drawn residues are identified and discussed in the text.

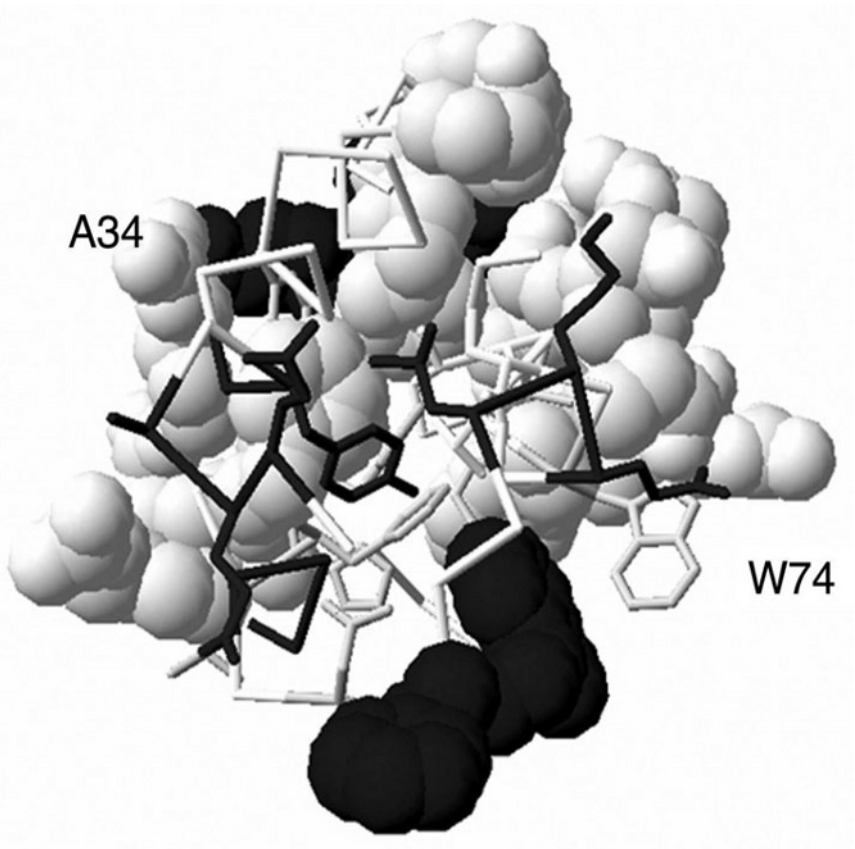


**Figure 2.** Structure of RAMP1, looking down on the N-terminus. Space filling residues shown in grey are in regions where deletions caused a major loss of CGRP- and AM-responsiveness as well as RAMP1 cell-surface expression. Space filling residues shown in black are in regions where deletions caused a major loss of RAMP1 cell-surface expression alone. Residues in line-drawn form in black are in regions where deletions altered AM responsiveness alone. Other line-drawn residues are identified and discussed in the text. Note how the residues implicated in AM-responsiveness are clustered.

epitopes that cause a major loss of both CGRP-responsiveness and RAMP1 cell-surface expression are predominantly clustered towards the C-terminal end of RAMP1. The epitopes that are involved in AM binding are found at the opposite end of the protein (Fig. 2).

A limited amount of work has been done to identify individual amino acids which are important for CGRP receptor function; collectively the studies suggest helices 2 and 3 of RAMP1 are most important for CLR recognition.[24] An alanine scan of residues 91-103 in helix 3 showed that F101A significantly impaired RAMP1 expression and CGRP binding, although the effect on CGRP-stimulated cAMP production was not so pronounced. In addition, F93A and Y100A also inhibited RAMP1 expression by over 50%. Both of these mutants also impaired CGRP binding as did F92[A] and H97A. Interestingly, L94A, whilst not altering total RAMP1 expression, significantly enhanced cell-surface expression. The authors suggested that L94 may impair CLR association by steric hinderance and this was relieved by the alanine substitution.[22] The RAMP1 crystal structure shows that the side chain of L94 faces away from the main helical bundle of the protein, so it could face towards a partner such as CLR. A more recent study concluded that Y66 of helix 2 and H97 were particularly important in promoting RAMP1 interaction with CLR, with F93 and F101 playing lesser roles.[24] These residues all cluster together and form an exposed surface (Fig. 1), consistent with them defining an interaction site for CLR.[23] Mutation of residues in helix 1, on the other face of the protein, had little effect on CGRP responsiveness or association with CLR.[24]

Based on inspection of the crystal structure, it has been suggested that the binding site for CGRP could be formed by a cluster of residues at the C-terminal end of helix 2 including R67, D71, W74, N78 and W84. A recent mutagenesis study has identified L69 and T73, both within this region, as being important for CGRP binding.[24] However, mutation of W74 does not affect CGRP binding[25] and deletion of N78 and surrounding amino acids does not impair CGRP-responsiveness.[22] Unlike the situation for AM binding, the deletion studies did not reveal any discrete area of RAMP1 that was specifically involved in CGRP recognition, rather than CLR recognition.[22] This suggests that the domains of RAMP1 that are involved in CLR recognition are intimately associated with those that recognise CGRP. In support of this, F93, close to the presumed CLR interaction site, appears to have a specific role in CGRP binding. The substitution F93I in RAMP1 (changing the residue to its equivalent in RAMP3), inhibited CGRP potency without materially altering cell-surface expression of the CLR/RAMP1 complex.[26] It has also been noted that the mutation A34E causes a small increase in CGRP potency; this residue is located towards the upper end of RAMP1 (Fig. 2).

As noted above, the deletion mutants suggest the region of RAMP1 involved in AM binding in the CGRP receptor is its upper portion, adjacent to the N-terminus. Mutagenesis has provided firm evidence that W74, on the edge of this region (Fig. 2) is in close proximity to bound AM.[26]

Very little is known about the regions of CLR involved in RAMP1 recognition. The extreme N-terminus seems to be important (CLR residues 23-60); if this is transferred to the PTH1 receptor, that acquires the ability to associated with RAMP1.[27] In other family B GPCRs, the equivalent of this is at least partially an alpha helix and is in proximity to bound ligands.[11-14]

### Antagonist Binding

A small number of nonpeptide antagonists have been identified for the CGRP receptor; the best characterised of these is BIBN4096BS.[28] This is an allosteric antagonist[29] that shows a very high selectivity towards primate CGRP receptors. The basis of this selectivity is due to the nature of the residue at position 74 of RAMP1; in primates it is a tryptophan but in rodents and many other species it is a lysine. It is a glutamic acid in RAMPs 2 and 3; AM1 and 2 receptors also show a low affinity for BIBN4096BS. Interestingly, substitution of the glutamic acid for tryptophan increases the affinity of RAMP3 for BIBN4096BS.[29] W74 is not directly involved in the binding of CGRP. Residues 37-63 of CLR are needed for high affinity RAMP binding; interestingly these are also the residues implicated in antagonist binding,[30] further suggesting that it is the extreme N-terminus of CLR that is in contact with RAMP1.

## OTHER ROLES OF RAMP1 IN THE CGRP RECEPTOR

### Receptor Chaperones

Neither CLR nor RAMP by itself can readily be expressed at the cell surface; it is only the complex that can be trafficked from the endoplasmic reticulum.[6] RAMP1 lacks any glycosylation site. Furthermore, the sequence QSKRT in the short C-terminal tail of RAMP1 acts as an intracellular retention signal; its removal results in CLR-independent transport of RAMP1 to the cell surface.[31] CLR is slightly less dependent on RAMP1 association to find its way to the cell surface and under some conditions, appreciable quantities can accumulate.[32] However, it seems that normally little CLR will be transported to the cell surface unless RAMP is present.[33] Furthermore, even if CLR reaches the cell surface in the absence of RAMP1, it is functionally inactive.[15,32]

## Glycosylation

Initially it was noted that CLR, when associated with RAMP1, was more heavily glycosylated than when associated with RAMP2. This led to speculation that the differential glycosylation was the cause of the different pharmacological profiles shown by the CGRP and AM1 receptors.[6] However, co-expression of mammalian CLR and RAMP1 in Drosophila Schneider 2 cells produced receptors with normal CGRP pharmacology in spite of the fact that the glycosylation of CLR was different to that seen in HEK293 cells.[34] Subsequent work has shown that the differences in glycosylation were artefacts due to the epitope tags carried by RAMP1 and RAMP2 as used in the initial experiements.[15] There is no evidence that RAMPs themselves modulate glycosylation of CLR or that its differential glycosylation is relevant to the CGRP/AM receptor phenotypes.

## Receptor Signalling and Internalisation

The C-terminal tail of RAMPs plays a significant role in modulating the G-protein coupling of amylin receptors, where the RAMPs are complexed to the calcitonin receptor.[35] Removal of the C-terminus of RAMP1 reduced the potency of CGRP at stimulating cAMP formation at the AMY1 receptor.[36] Similarly, deletion of the C-terminal tails of RAMP2 and RAMP3 altered the cell-surface expression and internalisation respectively of the AM1 and AM2 receptors. However deletion of the C-terminal tail of RAMP1 had little effect on any property of the CGRP receptor.[37,38] This is broadly consistent with the data of Steiner and coworkers, who showed that deletions of the first nine amino acids of the tail of RAMP1 had little impact on CGRP receptor function.[31] Thus it seems that the CLR:RAMP1 complex is resistant to modifications of the C-terminus of RAMP1. This may be linked with the presence of another peripheral protein which is a key part of the CGRP receptor, receptor component protein (RCP), which enhances coupling of the receptor to Gs.[39] Perhaps RCP substitutes for the C-terminal tail of RAMP1 in coupling to effector proteins.

The CGRP receptor is phosphorylated and internalised in clathrin-coated pits in an arrestin-dependent manner. RAMP1 remains associated with CLR during this process.[16,17] Although the complex is recycled to the cell surface after transient stimulation, it is degraded following sustained CGRP treatment. During the degradation, RAMP1 is destroyed at four times the rate of CLR.[40] The role of RAMP1 in the internalisation and recycling process appears largely passive; by contrast, a PDZ domain in RAMP3 ensures AM2 receptors are efficiently recycled, at least when expressed in the appropriate cell type.[41]

## RAMP EXPRESSION AND CGRP FUNCTION

Given that RAMP1 is a key part of the CGRP receptor, it might be predicted that changes in RAMP1 expression would influence CGRP receptor expression and hence the sensitivity of cells to the peptide, provided RAMP1 concentrations were limiting or, alternatively, an increase in its synthesis was coupled to that of CLR. Changes in CGRP receptor activity have indeed been reported as a consequence of altering RAMP levels.

RAMP1 knockout mice have elevated blood pressure, consistent with reduced CGRP receptor activity. Furthermore, CGRP shows a markedly reduced vasodilator activity in these animals and also has reduced activity to suppress pro-inflammatory cytokine production.[42] By contrast, RAMP1-overexpressing mice show enhanced plasma extravasation when CGRP is injected into the whisker pad.[43] This is particularly interesting as it demonstrates that over-expression of RAMP1 in the trigeminal system enhances CGRP sensitivity; CGRP acting on the trigeminal complex causes many of the symptoms of migraine. RAMP1 can be upregulated by hormones. Aldosterone treatment of mesenteric arteries from spontaneously hypertensive rats increased both CGRP sensitivity and RAMP1 expression but not CLR expression.[44] Interestingly, in cerebral arteries aldosterone enhances CLR expression but not RAMP1 and this is not associated with increased CGRP sensitivity.[45] Thus at least in some vascular beds, RAMP1 expression seems to limit CGRP receptor activity. In other vascular beds, parallel changes in CLR and RAMP1 expression have been reported. Both decrease during pre-eclampsia in fetoplacental vessels and there is also a loss in CGRP binding sites.[46] RAMP1 levels can increase in disease, in a manner consistent with changes in CGRP receptor activity. Thus in a rat model of chronic heart failure, RAMP1 expression increased in atria and ventricles.[47]

## CONCLUSION

RAMP1 is an essential component of the CGRP receptor. Its amino terminus forms a complex with the amino terminus of CLR to create a binding site for CGRP. It is also intimately involved in the binding of at least some CGRP antagonists. Changing RAMP1 expression can alter CGRP sensitivity. Thus targeting RAMP1 when it is complexed to CLR or manipulating its expression is very fruitful way of altering CGRP activity.

## NOTE ADDED AFTER PROOFS

Since this chapter was written in 2008/9, crystal structures of the extracellular domains of the CLR-RAMP1 complex with and without bound nonpeptide antagonists have been published.[48] Readers should consult more recent reviews for an up-to-date overview.[49, 50, 51]

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CHAPTER 3

# REGULATION OF GPCR TRAFFICKING BY RAMPs

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**Abstract:** AM and CGRP receptors undergo differential intracellular receptor trafficking upon ligand stimulation. Intracellular trafficking of CLR/RAMP receptor complexes is regulated by posttranslational modifications and protein-protein interactions that differ for each cell type. Recent evidence is accumulating to suggest that the RAMP isoform in complex with CLR may play a role in determining the intracellular trafficking and fate of ligand-stimulated receptor complexes. In this chapter, we will review the current literature on mechanisms of regulating receptor trafficking and roles that have been demonstrated for RAMPs in this regulation.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

G protein-coupled receptors (GPCRs) are known to convey environmental signals to the intracellular environment via heterotrimeric G proteins to affect the cellular behavior. Their form of regulation had in the past been thought of as short-term control, whereas more recently they have been shown to play roles in longer-term regulation of such physiological processes as proliferation, apoptosis, cellular migration and hypertrophy.[1,2] Their long-term regulation is partially a result of the desensitization and endocytosis processes and the role of these processes in the intracellular signaling. This attenuation of signaling and removal of the receptor from the plasma membrane can decrease the receptor numbers capable of signaling for minutes to hours, depending on the cell and receptor type. Desensitization of a receptor often signals for the removal of the receptor from the plasma membrane by the pinching off of the plasma membrane and internalization of the receptor, termed endocytosis (see Table 1 and Fig. 1). Many forms of regulation control these processes, some of which being phosphorylation of the receptor on the cytoplasmic face, interaction of the receptor with additional proteins (caveolin, arrestin molecules) and interaction of the receptor with the endocytic machinery (clathrin, dynamin, adaptor protein-2 (AP-2), etc.). Receptors, regardless of the type, are often

**Table 1.** Terminology for steps in GPCR lifecycle

| Term | Definition |
|---|---|
| Desensitization | Attenuation in receptor signaling in response to prolonged agonist exposure |
| Endocytosis | Uptake by a cell of material from the environment by invagination (infolding) of its plasma membrane |
| Internalization | Transportation of cells or soluble material into the cell via a vacuole/ vesicle |
| Resensitization | Return of unbound receptor to plasma membrane, ready for ligand stimulation and receptor signaling |
| Recycling | Targeting of receptor from endosome for return pathway to plasma membrane for continued signaling |
| Degradation | Targeting of receptor from endosome for pathway to lysosomes/ proteosomes to be degraded |
| Down-regulation | Trafficking of receptor for internalization and degradative pathway to promote decrease of available receptors at plasma membrane |

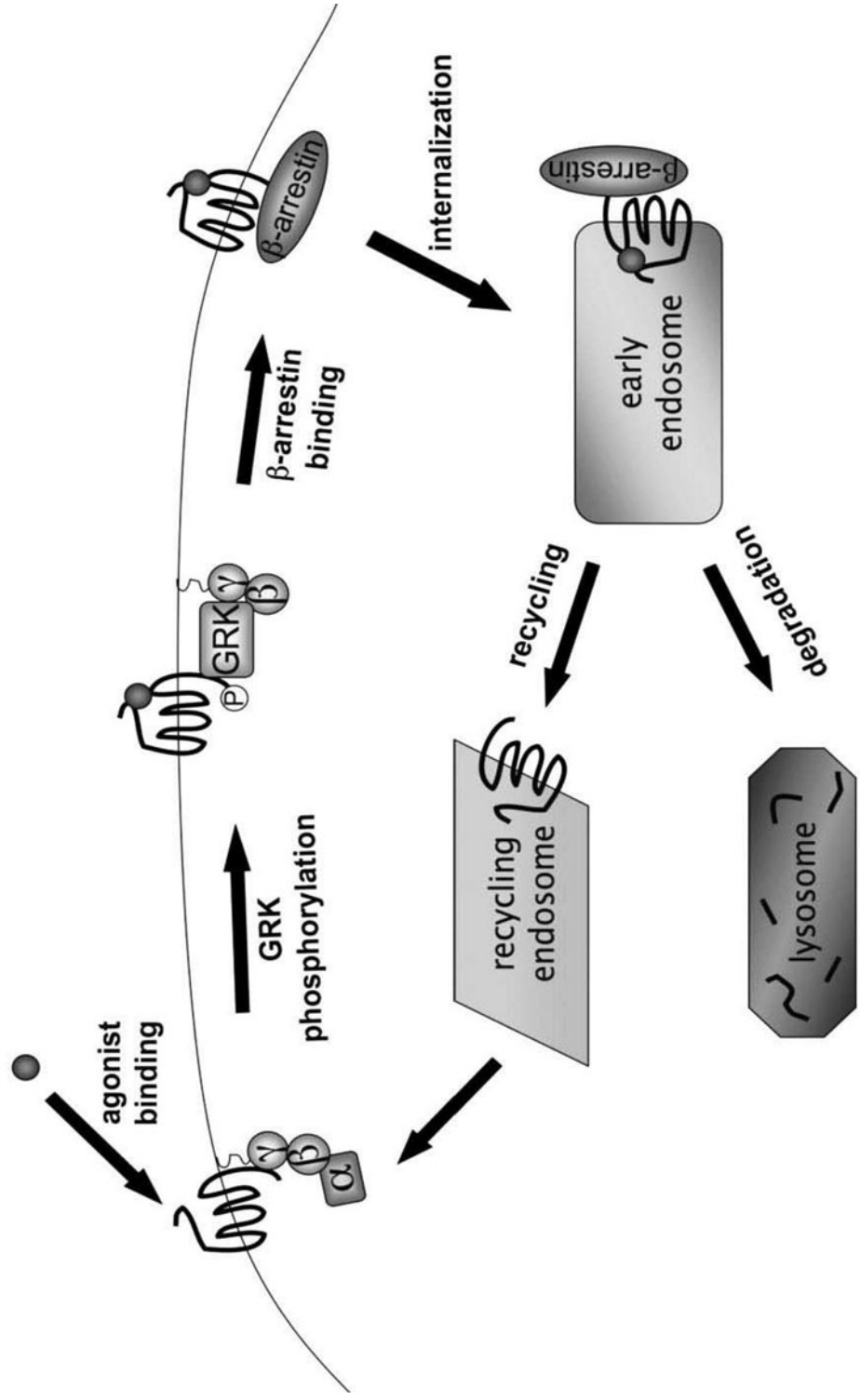


**Figure 1.** Depiction of GPCR lifecycle (modeled for prototypical GPCR lifecycle of the $\beta_2$-adrenergic receptor).

endocytosed into the early endosomes where their fate is determined. From this point receptors are targeted for recycling to the plasma membrane or sorted for degradation in the lysosomes. Protein-protein interactions and posttranslational modifications are being elucidated that serve as sorting signals from the early endosome, to target proteins for either recycling to the plasma membrane to promote receptor signaling or shuttling of the receptor to the lysosomes for proteolytic degradation and down-regulation of the receptor. The factors that determine the regulation of the GPCR lifecycle will be discussed in the following sections.

## REGULATION OF RECEPTOR DESENSITIZATION

A prolonged exposure of cells to a particular ligand results in an attenuation of responsiveness to subsequent stimulation with that ligand. This phenomenon is called desensitization, which can be classified as homologous or heterologous. Homologous desensitization is defined as a decrease in the response that is specific for the stimulated receptor. Because G-protein regulated kinases (GRKs) are activated and only phosphorylate GPCRs in the agonist-bound state, GRKs are capable of attenuating receptor signaling by homologous desensitization.[3,4] An attenuation of receptor signaling that is the result of second messenger signaling and in principle not specific for the activated receptor, is termed heterologous desensitization. Protein kinase A and C are kinases activated through second messenger signaling of GPCRs and therefore can activate heterologous receptor desensitization.[5]

The most comprehensive study of desensitization, to date, has been in elucidating the molecular mechanisms of this process for the β-adrenergic receptors.[4,6] Using concepts from the examination of desensitization of the β-adrenergic receptors, it has been shown in numerous membrane-bound receptor systems, particularly GPCRs, that phosphorylation is an integral step in the attenuation of receptor signaling, termed desensitization. Virtually all GPCRs thus far studied have specific serine, threonine and/or tyrosine amino acid residues in the third intracellular loop or C-terminus of the receptor that require phosphorylation for the efficient desensitization of the receptor. In most cases, phosphorylation of these key residues allows for interaction with nonvisual arrestins. This interaction disrupts the coupling of the receptor to the G-protein, thus halting signaling of the receptor through the G-protein uncoupling. In addition, β-arrestin interaction with the GPCR promotes clathrin-mediated receptor internalization.

The mechanism of desensitization of CLR has been studied in several cell lines to date. Desensitization of CLR was found to involve protein kinase A activation in rat mesangial cells (RMCs) stimulated with AM, SK-N-MC (neuroblastoma cell line) cells stimulated with CGRP and vascular smooth muscle cells (VSMCs) stimulated with CGRP.[7-9] Whereas, in HEK 293 cells, desensitization of the CGRP receptor (CLR-RAMP1) has been shown to be dependent on the activity of G-protein receptor kinase (GRK)-6.[10] In HEK 293 cells, CLR has been shown to be phosphorylated when stimulated with agonist (AM or CGRP) via in vivo phosphorylation assays.[11] Given the published data for the β-adrenergic receptor and additional GPCRs supporting a phosphorylation-dependent mechanism for receptor desensitization and the above-described studies with CLR, it is hypothesized that phosphorylation of CLR is required for receptor desensitization after prolonged agonist exposure.

The mechanism of desensitization of CLR differs in microvascular endothelial cells for the endogenous CGRP and AM receptors. Intriguingly, both AM and CGRP receptors desensitize with prolonged exposure to either ligand, but only AM receptors internalize in conjunction with desensitization.[12] CGRP receptor desensitization in the absence of a loss of cell surface expression suggests GRK-mediated mechanism of desensitization.[13] The work of Aiyar et al, demonstrating a role for GRK6 in the CGRP-mediated desensitization of the porcine CLR in HEK 293T cells, supports this hypothesis.[10] A detailed analysis of the protein-protein interactions of the endogenous AM and CGRP receptors may provide insight into the differential receptor desensitization patterns observed in response to AM or CGRP.

With respect to physiological effects of the desensitization process, the inhibition of the process of desensitization for particular GPCRs has been shown to elicit protective therapeutic effects. Lefkowitz et al. have shown that the inhibition of β-adrenergic receptor kinase (kinase that phosphorylates the β-adrenergic receptor to cause desensitization) in the heart can delay the development of heart failure in multiple animal models, in some cases even restoring cardiac function.[14,15] Others have also shown inhibition of desensitization of the μ-opioid receptor to be beneficial in preventing morphine tolerance.[1,16] Because of the potential therapeutic effect of prolonged AM signaling in a system where it exerts protective effects, understanding the regulation of the desensitization process for the AM receptor is crucial. Moreover, in disease states like inflammation, diabetes and cardiovascular disease where prolonged AM or CGRP signaling is deleterious, elucidating the mechanisms that reduce signaling will be crucial for therapy development.

## REGULATION OF RECEPTOR INTERNALIZATION/ENDOCYTOSIS

Predominantly, endocytosis of G-protein coupled receptors is enhanced by agonist binding, whereas many nutrient receptors, such as the transferrin and LDL receptors, are constitutively endocytosed regardless of ligand occupancy status.[3,17-20] Clathrin-coated vesicles mediate the most well characterized pathway for receptor internalization. Often, clathrin-mediated endocytosis of mammalian GPCRs requires agonist stimulation and interaction with β-arrestin molecules. The β-arrestin proteins not only disrupt G-protein coupling with the receptor, but also serve as adaptors to link the receptor to the endocytic machinery.[21,22] Clathrin-mediated endocytosis generally also requires the activity of dynamin GTPases for proper vesicle formation and cleavage from the plasma membrane.[23]

An additional pathway for receptor internalization is now emerging. Published data now confirms that receptors are internalized via noncoated vesicles, such as the flask-shaped caveolae and other pinocytic mechanisms.[18,23] Caveolin-mediated internalization also requires the activity of dynamin, but internalization mechanisms independent of dynamin have also been suggested.[23,24] Caveolin-mediated endocytosis involves receptor complex localization in a lipid raft domain enriched in caveolin proteins, termed caveolae. Internalization of receptors by different mechanisms may be determined by the phosphorylation state of the receptor. The β1-adrenergic receptor has been demonstrated to undergo agonist-stimulated internalization via caveolae when phosphorylated by protein kinase A and internalization by clathrin-coated pit endocytosis when phosphorylated by GRKs during the desensitization process.[25] These studies may shed light on the study by Nikitenko et al in microvascular endothelial cells where both CGRP or AM stimulation results in desensitization of the CGRP or AM receptors, but only the AM receptors endocytose upon prolonged ligand stimulation.[12] Additional studies investigating the mechanism of endocytosis of the AM and CGRP receptors may define a role for the RAMP proteins in the differential response to prolonged AM or CGRP exposure.

In addition to phosphorylation state, protein-protein interactions can regulate the internalization pathway of a GPCR. NHERF-1, a PSD-95/Discs-large/ZO-1 homology (PDZ) domain-containing protein has been shown to associate with several GPCRs to regulate their agonist-mediated endocytosis. NHERF-1 is characterized as a 55 KDa protein containing two tandem PDZ domains, Type I and Type II domains, respectively.[26] NHERF-1 has also been shown to associate with members of the ezrin/radixin/moesin family of actin-binding proteins and thus has the ability to link PDZ domain-interacting proteins to the actin cytoskeleton.[27]

Several studies have shown NHERF-1 to play differing roles in various cellular processes, including receptor trafficking.[27,28] NHERF-1 has been demonstrated to bind the extreme COOH terminus of several G-protein coupled receptors (GPCRs) via its PDZ domains, namely the $\beta_2$-adrenergic receptor, the κ-opioid receptor, PTH-R and the P2Y purinergic receptor.[29,30] In addition to GPCRs, NHERF-1 has also been shown to associate with the epidermal growth factor receptor (EGFR), a receptor tyrosine kinases (RTK). While utilizing different mechanisms, NHERF-1 interaction with both RTKs and G-protein-coupled receptors tends to enhance the abundance of receptors at the plasma membrane.

Similarly, NHERF-1 interaction with the AM2 receptor is essential to tether the receptor-complex at the membrane, the absence of which leads to internalization of the receptor complex.[31] In the presence of NHERF-1, although the AM2 receptor (CLR/RAMP3) undergoes desensitization, the internalization of the receptor complex is blocked. Mutational analysis indicated that RAMP3 and NHERF-1 interact via a PDZ Type I domain on NHERF-1 and a PDZ recognition motif on the extreme C-terminus of RAMP3. The PDZ recognition motif is not present on RAMP1 or 2 and thus, NHERF-1 is incapable of blocking the endocytosis of the AM1 or CGRP receptors upon ligand stimulation. Mutation of the ezrin/radixin/moesin (ERM) domain on NHERF-1 indicated that NHERF-1 inhibits CLR/RAMP3 complex internalization by tethering the complex to the actin cytoskeleton. Studies in a primary culture of human proximal tubule cells endogenously expressing the CLR/RAMP3 complex and NHERF-1 confirms that the CLR/RAMP3 complex desensitizes but is unable to internalize upon agonist stimulation.[31] An observation lending further support for RAMP3-NHERF-1 PDZ domain interactions reducing AM2 receptor endocytosis upon agonist exposure is that, deletion of the C-terminal tail of RAMP3 dramatically increased the rate of internalization of the CLR/RAMP3 receptor complex in HEK 293T cells.[32] Taken together, these studies suggest a critical role for protein-protein interactions and NHERF-1 in regulation of AM2 receptor internalization and maintenance of receptor numbers at the plasma membrane.

## REGULATION OF RECEPTOR RECYCLING VS DEGRADATION

Agonist-induced endocytosis is a means of regulating signaling for a multitude of membrane-bound receptors, particularly for G-protein coupled receptors. For some receptors in distinct cell backgrounds, internalization is a means of rapid recycling, characterized by dephosphorlyation and dissociation from agonist in intracellular vesicles for restoration at the plasma membrane in a functional state to achieve resensitization. On

the other hand, some receptors that are internalized and targeted to late endosomes and lysosomes experience proteolytic degradation, thus promoting down-regulation of the receptor at the plasma membrane and attenuation of receptor signaling. Factors influencing the sorting of receptors in the early endosome are largely unknown, but some of the critical players are beginning to be identified for the GPCRs. Receptor ubiquitination, interaction of the receptor with PDZ domain-containing proteins and/or interaction of the receptor with additional, newly-identified proteins has been shown to be required in some GPCR systems for efficient targeting of the GPCR for either degradation or recycling pathways.[33-35]

Several studies have now established that RAMPs initially interact with their receptor partners in the ER/Golgi and maintain this association throughout the lifecycle of the receptor complex. The RAMP and receptor are trafficked together through the endocytic pathway to the recycling endosome or lysosome after agonist activation of the receptor.[11,36-38] These findings provide support for a role for RAMPs in determining the post-endocytic fate of CLR.

Much effort is currently being placed on the signal that sorts the internalized receptors for recycling or degradation. Ubiquitination of GPCRs has recently been shown to contribute to the sorting fate of the receptors for degradation. Ubiquitination is a process that involves the addition of multiple ubiquitin molecules on targeted lysine residues of a protein marked for degradation by an ubiquitinating enzyme complex.

A recent study by Cottrell et al reports that the CLR/RAMP1 receptor complex is degraded in the lysosomes after prolonged CGRP agonist exposure. Interestingly, in HEK 293 and SK-N-MC cells, the CGRP receptor is recycled upon endocytosis from transient ligand stimulation, while a prolonged ligand exposure targeted the CGRP receptors for down-regulation.[39] Although both CLR and RAMP1 are degraded in the lysosomal compartment, RAMP1 is reported to be degraded approximately 4-fold more rapidly than CLR. Western blotting to detect ubiquitination of CLR or RAMP1 suggests that a ubiquitin-independent mechanism is responsible for the sorting of the CLR/RAMP1 receptor complex for degradation.[39] In addition, previous studies by Kuwasako et al demonstrate that deletion of the C-terminal tail of all three RAMP isoforms, containing conserved lysine residues, was unable to promote CLR/RAMP receptor complex recycling.[32] These studies are the first to investigate the ubiquitin status of CLR/RAMP complexes targeted for degradation. More work in this field may begin to elucidate the differential sorting for CLR-RAMP receptor complexes after ligand-stimulated endocytosis.

It has been shown in several GPCR systems that protein-protein interactions, more specifically with PDZ domain containing proteins,

are responsible for the efficient targeting of the receptor after internalization.[30,40-42] This is a relatively new area of research in the GPCR field, but it is currently thought that the presence of a PDZ recognition sequence on the C-terminus of the receptor is the sorting signal for the interaction of the receptor with the PDZ domain-containing protein and targeting of the receptor for recycling to the plasma membrane from the early endosome. Recycling of the β2-adrenergic receptor (β2-AR) is dependent on the interaction of β2-AR, via its PDZ recognition motif, with a protein called N-ethylmaleimide sensitive factor (NSF).[41,42] β2AR interacts with NSF via a PDZ Type I recognition motif (-DSLL) at its extreme C-terminus that enhances the recycling of the β2AR after agonist-stimulated receptor internalization. While CLR, RAMP1 and RAMP2 do not contain PDZ recognition motifs, the RAMP3 isoform contains a canonical Type I PDZ recognition sequence at its extreme C-terminus. This raises the possibility that PDZ domain interactions may regulate AM2 receptors via protein-protein interactions with RAMP3 and regulatory proteins.

A report from Bomberger et al demonstrated a role for NSF in the sorting of the AM2 receptor after internalization for a recycling pathway. In this case, NSF's interaction with RAMP3 directs the trafficking of a receptor from the sorting endosome.[38] In HEK293T cells, the transiently expressed CLR/RAMP complex undergoes agonist-stimulated desensitization and internalization and fails to resensitize (i.e., degradation of the receptor complex). Co-expression of N-ethylmaleimide-sensitive factor (NSF) with the CLR/RAMP3 complex, but not CLR/RAMP1 or CLR/RAMP2 complex, altered receptor trafficking to a recycling pathway. Mutational analysis of RAMP3, by deletion and point mutations, indicated that the PDZ recognition motif of RAMP3 interacts with NSF to cause the change in trafficking. The role of RAMP3 and NSF in AM2 receptor resensitization was confirmed in rat mesangial cells, where siRNA-mediated knockdown of RAMP3 and pharmacological inhibition of NSF both resulted in a lack of receptor resensitization/recycling after agonist-stimulated desensitization. These results indicate a novel function for RAMP3 in the post-endocytic sorting of the AM-R and suggest a broader regulatory role for RAMPs in receptor trafficking.

A recent report by Kuwasako et al in HEK 293T cells failed to repeat the redirecting of CLR/RAMP3 receptor complex to a recycling pathway with NSF overexpression.[32] The discrepancy may be due to different transfection protocols or expression of a bovine vs human CLR isoform, a factor known to result in altered pharmacological properties of the CLR/RAMP receptor complex.[43]

As described, the fate of the GPCR is quite variable after agonist-induced internalization, with multiple GPCRs shown to both recycle and degrade,

depending on the cell type and agonists interacting with the receptor. In the case of CLR, the receptor fate differs, depending on the cell type where the receptor is expressed. It has been shown that CLR/RAMP complex will degrade upon prolonged agonist exposure and receptor endocytosis in HEK 293 cells and Rat2 fibroblast cells, while in rat mesangial cells (RMCs) the receptor complex is effectively recycled after internalization.[7,37] Cellular background plays a major role in determining receptor complex fate after internalization in large part because of critical protein-protein interactions that act to regulate the intracellular trafficking of a particular receptor. An elegant study by Padilla et al recently demonstrated that the metalloendopeptidase, endothelin-converting enzyme-1 (ECE-1), is a protein that regulates the endocytic recycling of the CLR/RAMP1 complex.[44] ECE-1 acts at the level of the early endosome to degrade CGRP, thus allowing the unbound receptor complex (CLR/RAMP1) to recycle back to the plasma membrane. ECE-1 inhibition or siRNA-mediated knockdown confines CLR/RAMP1 to the early endosomes bound to CGRP and β-arrestin2, thus preventing the recycling and resensitization of the receptor complex.[44] Further studies are needed to delineate if ECE-1 plays a role in the intracellular trafficking of the AM receptors, as well, or if this protein interacts specifically to degrade neuropeptides and therefore is exclusive for the CLR/RAMP1 receptor complex.

## CONCLUSION

Given the multitude of differences in structure and function of GPCRs and different signaling mechanisms through which they operate, it is clear that the pathways by which they desensitize and down-regulate are also quite different. The presence of the RAMPs in the AM and CGRP receptor complexes adds another layer of complexity and it appears to provide an additional mechanism for regulation of intracellular trafficking. This chapter has highlighted several protein-protein interactions and posttranslational modifications that have been reported to regulate the intracellular trafficking of the AM or CGRP receptors (Fig. 2). For these reasons, it is critical to understand and clarify the mechanisms and pathways that govern this important process in the signaling of a cell. The protective role the CLR-AM system has been shown to play in cardiorenal and pulmonary pathophysiological situations and possible role of the CLR-CGRP system in certain clinical conditions, makes elucidating the desensitization and down-regulation pathways of this receptor system of great importance.

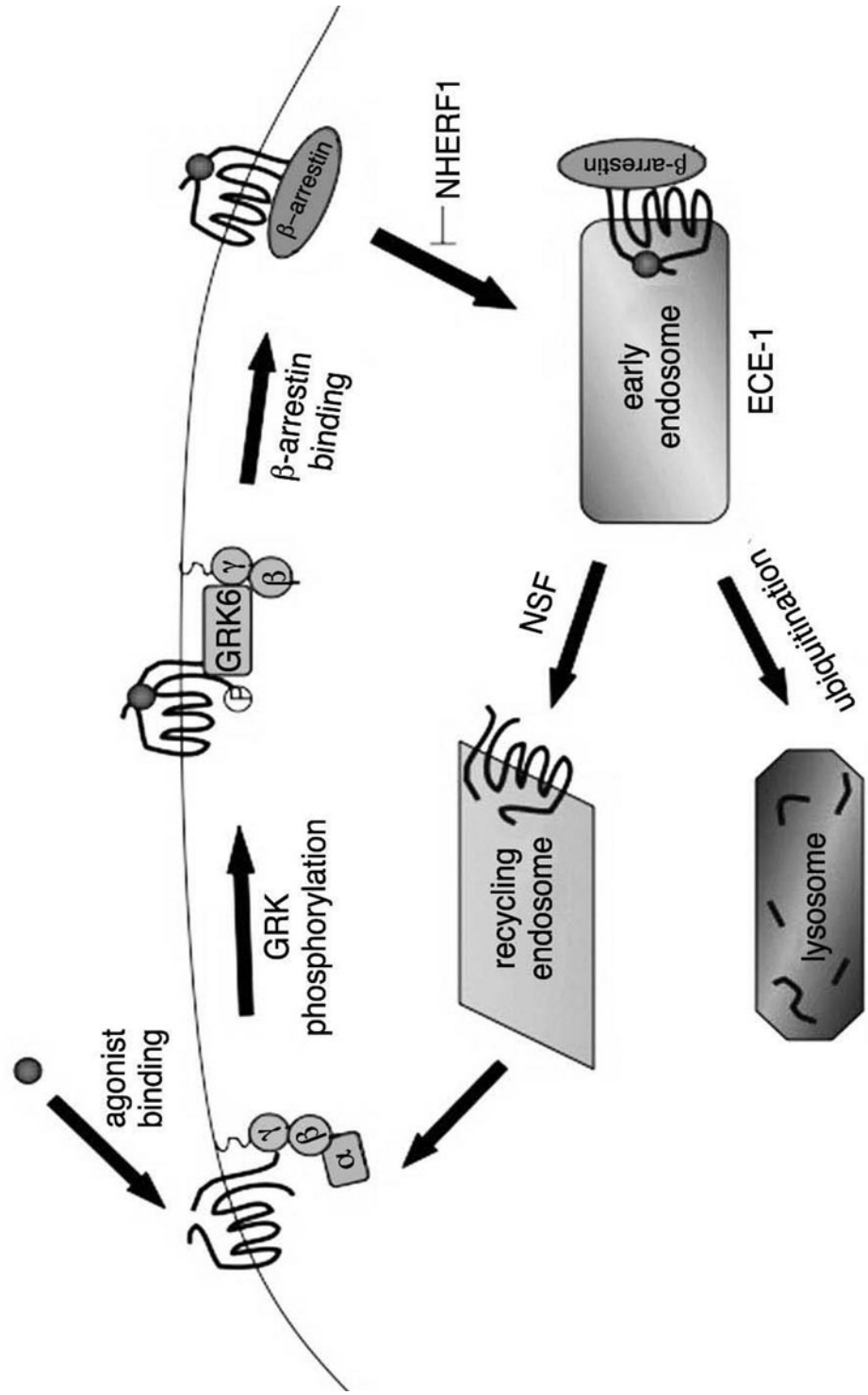


**Figure 2.** CLR lifecycle regulation.

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CHAPTER 4

# REGULATION OF CALCIUM SENSING RECEPTOR TRAFFICKING BY RAMPs

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**Abstract:** As mentioned earlier in this book, RAMPs were identified as proteins escorting the Calcitonin Receptor-Like Receptor (CRLR) to the plasma membrane (PM) to generate either CGRP (when associated with RAMP1), or adrenomedullin receptors (when associated with RAMP2 or RAMP3). Some years after this initial discovery, it was established that RAMPs can accompany four additional class B G Protein-Coupled Receptors—GPCRs—(PTH1, PTH2, glucagon receptor and VPAC1) to the PM.[1] By demonstrating that the sorting traffic of the Calcium Sensing Receptor (CaSR), a class C GPCR, is positively modulated by RAMP1 and RAMP3,[2] our data extended the concept of RAMPs as escorting molecules to another class of GPCRs.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION TO THE CaSR, THE PROTOTYPE OF CLASS C GPCR INTERACTING WITH RAMPs

The CaSR is a GPCR that plays a crucial role in extracellular calcium signalling and homeostasis.[3] The CaSR detects fluctuations in free ionized extracellular calcium concentration and triggers signalling cascades to constantly readjust levels of circulating calcium. Inactivating or activating mutations in the casr gene in humans are responsible for hypercalcemia or hypocalcemia, respectively,[3-6] illustrating the mandatory role of the CaSR in the maintenance of calcium homeostasis. Besides these well-established roles, the CaSR also participates in cell-type specific processes that involve local changes or gradients in extracellular calcium, such as stem cells homing[7] and neuronal processes outgrowth.[8]

Structurally, the CaSR is a class C GPCR closely related to metabotropic gamma-aminobutyric acid ($GABA_B$) and glutamate (mGlu) receptors.[3,9] In common with these receptors, the CaSR contains a large extracellular N-terminal that includes a venus flytrap domain required for agonist binding.[10] The receptor is present as CaSR/CaSR homodimers at the cell surface.[11] The N-terminal domains project into the extracellular space and can detect small increases in extracellular calcium concentration that activate the receptor complex and trigger subsequent signalling cascades.

To function correctly, the CaSR must be efficiently delivered to the PM and there is accumulating evidence to show that several loss of function CaSR mutants are subject to compromised forward trafficking[12-16] (for a recent review, see ref 17).These receptors are either not expressed or only poorly expressed at the PM and are therefore defective in mediating systemic calcium homeostasis.

## SEP-CaSR: A TOOL TO TRACK CaSR TRAFFIC

To get insight into the trafficking properties of the CaSR, we fused the GPCR to the pH sensitive GFP variant named Super Ecliptic pHluorin (SEP).[2] SEP is a reporter molecule that is essentially nonfluorescent at acidic pH values ($\leq$6.0) and whose brightness increases with pH values up to 8.5.[18] Vesicular compartments of the sorting pathway have an acidic lumen, with the exception of the ER that has a relatively neutral luminal pH of ~7[19] (Fig. 1A). SEP was inserted in the extracellular N-terminal domain of CaSR, which orientates inside the vesicle lumen (Fig. 1A). Hence, SEP-CaSR is visible in the ER and becomes less visible during progress through the increasingly acidic compartments along the sorting pathway (Fig. 1A). Once the receptor is surface expressed the SEP tag is exposed in the extracellular space and the

fluorescence intensity of surface expressed SEP-CaSRs is dictated by the pH of the extracellular media (Fig. 1A).

Accordingly, in confocal imaging experiments of HEK293 cells transiently expressing SEP-CaSR there is a marked and reversible decrease in SEP-CaSR fluorescence at the PM in extracellular media at pH 6 compared to the fluorescence obtained at pH 7.4[2] (Fig. 1B). At pH 7.4 transient application of $NH_4Cl$ (that collapses all membrane proton gradients and equilibrates all compartments to the extracellular pH values) leads to an increase in total fluorescence due to the normally nonfluorescent intracellularly SEP-CaSR becoming visible (Fig. 1B). In parallel, we determined that CaSR faculties to form homodimers and to recruit transduction cascade (such as calcium mobilization) were unaffected by the tagging with SEP.[2] Thus, SEP-CaSR can be used as a tool to track the trafficking of the receptor at the plasma membrane. Similar strategies using the same SEP reporter have subsequently been successfully applied to study the traffic of other GPCRs such as mGluR7[20] and the cannabinoid receptor CB1.[21]

## THE CaSR IN EXPRESSED AT THE SURFACE OF HEK293 BUT ABSENT FROM COS7 CELL SURFACE

In contrast to HEK293 cells, when SEP-CaSR was expressed in COS7 cells, another widely used clonal cell line, no surface expression of the receptor was detected by immunocytochemistry, biotinylation experiments or SEP fluorescence (Fig. 1C).[2] Nevertheless, SEP-CaSR was correctly expressed in COS7 cells and displayed a high level of fluorescence within the ER of these cells suggesting that SEP-CaSR is efficiently expressed in COS7 cells but that it is not able to exit the ER. Supporting this hypothesis, the monomeric fully glycosylated form of the receptor detected in HEK293 cells was missing in COS7 cells extracts, indicating incomplete glycosylation.[2]

Taken together, these results suggested that the discrepancy in the expression of CaSR at the PM is the consequence of different ER sorting mechanisms in the two cell types. A likely explanation is that a factor positively regulating CaSR traffic in HEK293 cells was absent from COS7 cells. Inspection of the available literature revealed few identified proteins that escort GPCRs to the PM. Of those that have been determined, RAMPs were attractive candidates as the potential permissive factor. RAMPs escort the CRLR[22] and have been implicated in the traffic of several class B GPCRs.[1,23] Most intriguingly, RAMPs are present in HEK293 cells[22] but appear to be absent in COS7 cells.[24] However, since HEK293 and COS7 cells have been passaged thousands of times in hundreds of different labs there will now be divergence between the properties of the clonal cell lines used worldwide.

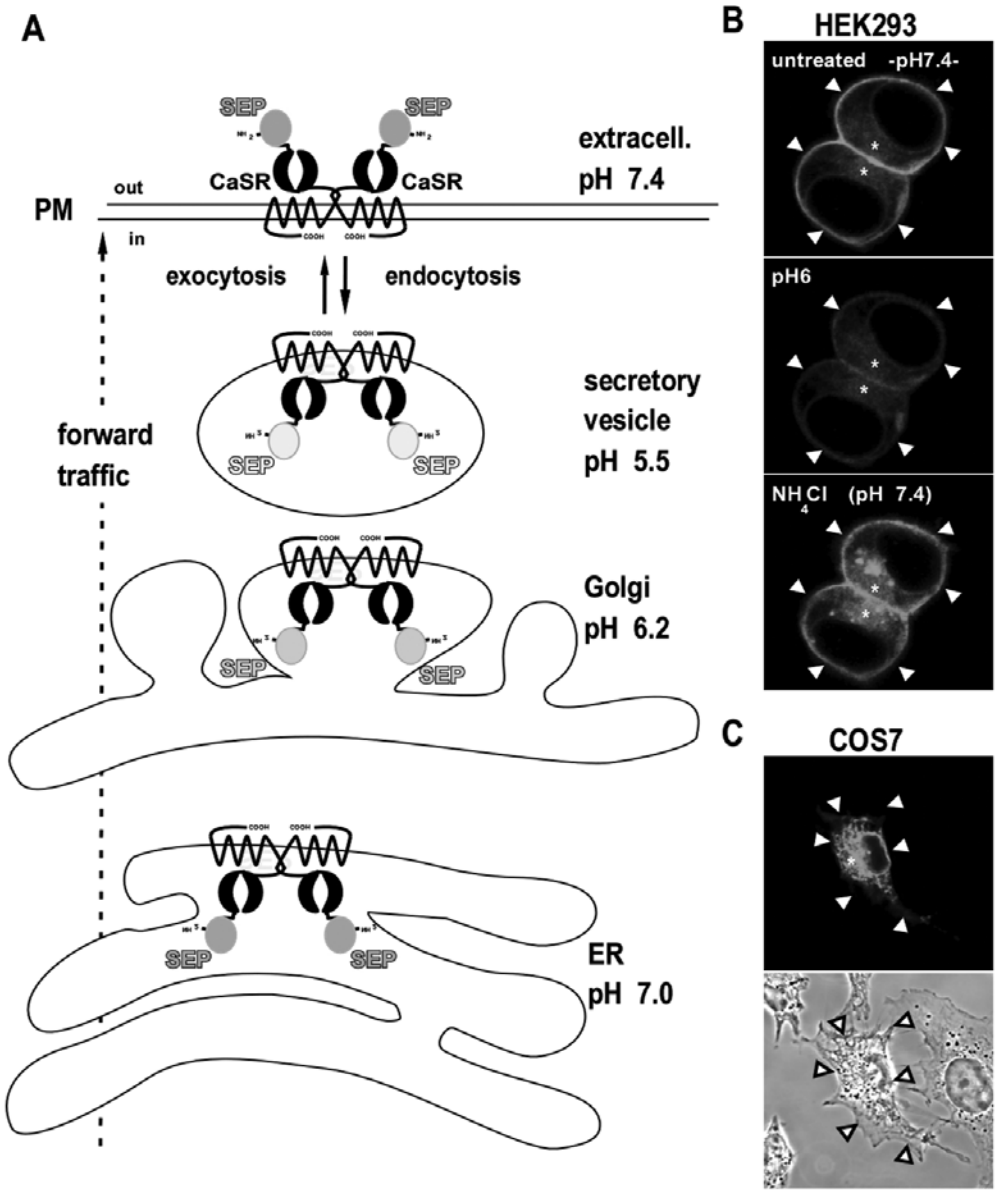


**Figure 1.** Super-Ecliptic pHluorin-tagged CaSR (SEP-CaSR), a tool to track CaSR cell surface expression and traffic. A) Theoretical levels of SEP-CaSR fluorescence at different pH levels in the compartments of the sorting pathway. Tagging with SEP allows real time visualisation of bulk endocytosis and exocytosis by measuring changes in fluorescence at the plasma membrane. B) Images obtained during live confocal imaging revealing the distribution of SEP-CaSR fluorescence in two cultured HEK293 cells transfected with SEP-CaSR. Note that the fluorescence is mainly visible at the plasma membrane (indicated by arrowheads) at an extracellular pH 7.4. Figure legend continued on following page.

**Figure 1,** continued from previous page**.** At pH 6, a decrease in fluorescence is observed as surface SEP-CaSR flourescence is eclipsed. By contrast, application of a solution at pH 7.4 containing $NH_4Cl$ (50 mM), which rapidly equilibrates intracellular pH levels, causes a sharp increase as all the SEP-CaSR fluorescence in the cell (marked by asterisks) is revealed. C) SEP-CaSR is absent from the surface of transfected COS7 cells. A transmitted light image shows the location of the plasma membrane. Reproduced from: Bouschet T et al. Trends Pharmacol Sci 2008; 29(12):633-639;[17] ©2008 with permission from Elsevier.

Importantly, we determined that our COS7 cells lack all RAMPs whereas our HEK293 cells expressed solely RAMP1.[2]

## RAMP1 AND RAMP3, BUT NOT RAMP2, PROMOTE CELL SURFACE DELIVERY OF THE CaSR

To test for the hypothesis that RAMPs regulate CaSR traffic, COS7 cells were cotransfected with SEP-CaSR and RAMP1, RAMP2 or RAMP3 (or with a control plasmid) and the SEP-CaSR surface expression was measured by biotinylation. SEP-CaSR was undetectable at the cell surface in COS7 cells coexpressing the receptor and a control plasmid or RAMP2 but there was a robust CaSR surface translocation upon expression of either RAMP1 or RAMP3. Consistent with these biotinylation data, surface labelling of SEP-CaSRs with anti-GFP antibody (that also recognizes SEP) showed that RAMP1 and RAMP3, but not RAMP2, promotes PM expression of SEP-CaSR. Thus, RAMP1 or RAMP3 are sufficient to promote surface expression of CaSR. Furthermore, the fact that RAMP2 is ineffective indicates specificity between the RAMPs for CaSR translocation to the surface. This specificity likely depends on the receptor type since, for example, all RAMPs interact with the CRLR[22] whereas only RAMP2 interacts with the glucagon receptor.[1]

## RAMPs AND CaSR FORM A COMPLEX ALONG THE SORTING PATHWAY

RAMPs and their GPCRs partners form complexes that originate in the ER and Golgi apparatus. These complexes are maintained during translocation to the cell surface, agonist activation and internalisation.[25,26] The extent of RAMP and SEP-CaSR colocalisation was determined by immunocytochemistry and confocal imaging. CaSR and RAMP1 or RAMP3 colocalised inside the cell and at the cell surface suggesting that RAMPs and CaSR may form a complex. Indeed, CaSR and RAMP1 or CaSR and RAMP3 co-immunoprecipitate although it remains to be determined whether this interaction is direct or occurs through a third common partner. In RAMP-lacking COS7 cells, CaSR locates in the ER and is with negligible levels present in the Golgi apparatus.

However, in COS7 cells transfected with RAMP3, the GPCR and RAMP3 colocalize both in the ER and in the Golgi and is fully glycosylated to a mature form at the cell surface.

These results suggest that RAMP3 and the CaSR associate in the ER and that RAMP3 permits the transit of the receptor to the Golgi where it acquires full glycosylation. As we detected some colocalisation between RAMP3 and CaSR at the cell surface, it is likely that the complex is maintained all along the sorting pathway, from the ER to the surface.

## CONCLUSION

Gain of function experiments in which RAMP1 or RAMP3 were coexpressed with the CaSR in RAMP-deficient COS7 cells demonstrate that RAMPs are sufficient to restore the cell surface targeting of the GPCR. In the converse loss of function experiments in which RAMP1 was suppressed from HEK 293 cells using RNA interference, the forward trafficking of the CaSR was impaired.[2] Thus, we conclude that RAMPs are both necessary and sufficient for the traffic of the CaSR to the PM. A model of RAMPs mechanisms of action on CaSR sorting traffic assembled from these results is shown in Figure 2.

However this model remains incomplete and further investigation will be required to determine if RAMPs are involved in the traffic of endogenously expressed CaSR in functionally appropriate cells. It is possible that the level of expression of RAMPs determines the presence or absence of functional CaSRs; in other words, CaSRs may be retained or released from the ER depending on the level and type of available RAMP. The CaSR and RAMP1 or RAMP3 are co-expressed in kidney and brain,[22] two organs where the CaSR plays a crucial role.[8,27,28] However, the expression of RAMPs in other cell types where CaSR plays an important physiological role, such as stem or parathyroid cells remains to be determined. Similarly, the degree of CaSR surface expression could also be affected by marked changes in RAMP expression during patho-physiological conditions.[29] In particular, RAMP1 mRNA is dramatically increased in an obstructive nephropathy model.[30] The functional consequences of this increase are unknown but may lead to a rise

**Figure 2,** viewed on next page**.** Proposed model for RAMP regulation of CaSR trafficking. A) In the absence of RAMPs (COS7 cells), the CaSR is retained (stop sign) in the endoplasmic reticulum (calnexin-positive compartment) in its incompletely processed, core-glycosylated form. B) By contrast, in cells expressing RAMP endogenously (such as HEK293 cells), or exogenously (such as RAMP-transfected COS7 cells), the CaSR in association with RAMP1 or RAMP3 bypasses the ER retention and reaches the Golgi apparatus (giantin-positive compartment) where it is terminally glycosylated before being delivered to the cell surface. Figure reproduced from: Bouschet T et al. J Cell Sci 2005; 118(Pt 20):4709-4720;[2] ©2005 with permission from the Company of Biologists.

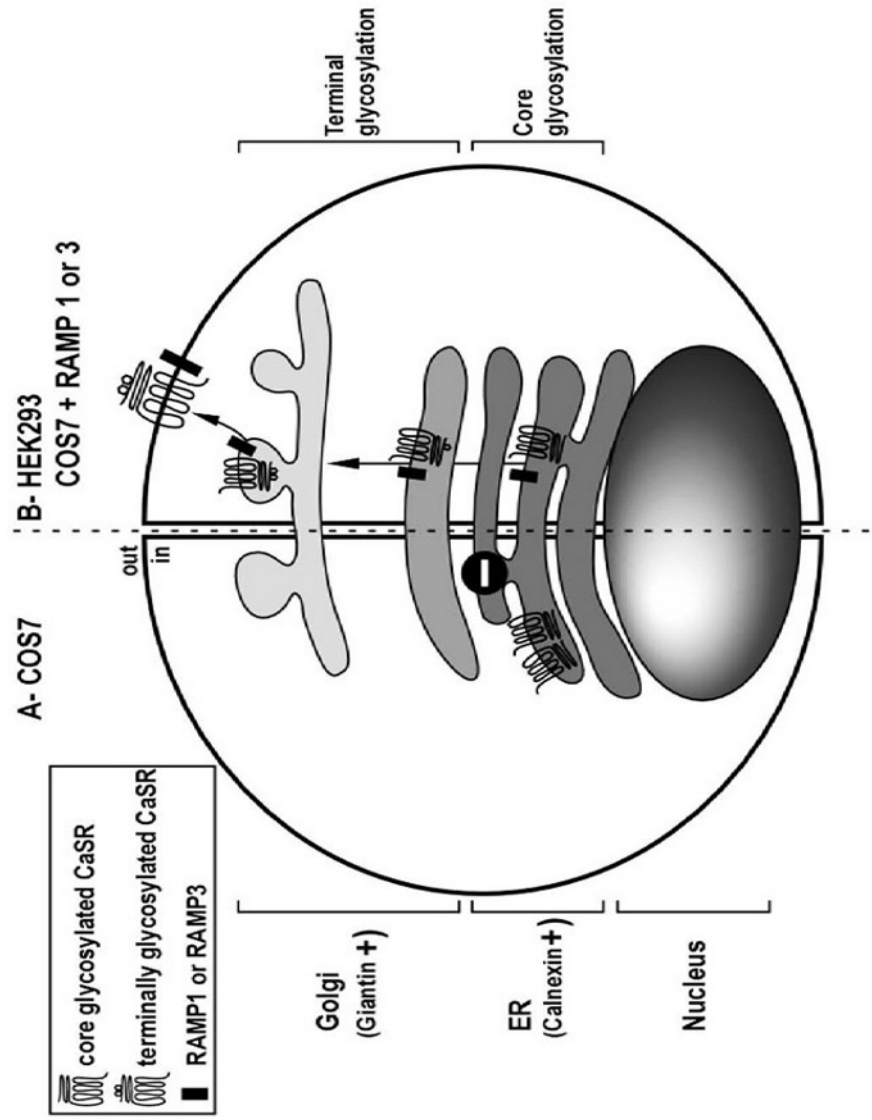


**Figure 2**. See legend on previous page.

in surface expression of the CaSR, which is furthermore important for renal physiology. Similarly, the expression of RAMP1 and RAMP3 is increased in rats with chronic heart failure.[31] The CaSR is expressed in cardiac tissue and myocytes where its activation triggers intracellular calcium mobilisation.[32]

The roles of RAMPs in the targeting and asymmetrical localisation of the CaSR in polarised cells[33] also remain undefined. RAMPs may also influence the pharmacological properties of the receptors. Given that the CaSR responds to multiple agonists in addition to calcium including spermine or amino-acids,[4] RAMPs could potentially alter the ligand potency and/or selectivity analogous to their roles in CRLR pharmacology.

Thus, future elucidation of the roles of RAMPs in CaSR signalling, pharmacology and trafficking will provide insight into the mechanisms regulating CaSR functions and therefore could lead to the development of therapeutic strategies to manipulate surface functional expression of the CaSR in diseased cells.

Finally, more exploration is required to determine whether other class C GPCRs interact with RAMPs or if the CaSR is an isolated planet in this galaxy of receptors.

## ACKNOWLEDGEMENTS

We thank the Wellcome Trust and the BBSRC for financial support.

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CHAPTER 5

# UNDERSTANDING RAMPs THROUGH GENETICALLY ENGINEERED MOUSE MODELS

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**Abstract:** The family of Receptor Activity Modifying Proteins (RAMPs) consists of three members, RAMP1, 2 and 3, which are each encoded by a separate gene and have diverse spatiotemporal expression patterns. Biochemical and pharmacological studies in cultured cells have shown that RAMPs can modulate several aspects of G receptor (GPCR) signaling, including receptor trafficking, ligand binding affinity, second messenger signaling and receptor desensitization. Moreover, these studies have shown that RAMPs can interact with several GPCRs other than the canonical calcitonin receptor-like receptor (CLR), with which they were first identified. Given these expanding roles for RAMPs, it becomes interesting to question how these biochemical and pharmacological properties bear significance in normal or disease physiology. To this end, several gene targeted knockout and transgenic models have been generated and characterized in recent years. Fortunately, they have each supported important roles for RAMPs during embryonic development and adulthood. This chapter provides a comprehensive overview of the most recent findings from gene targeted knockout mouse models and transgenic over-expression models, and gives special consideration to how comparative phenotyping approaches and conditional deletion strategies can be highly beneficial. In the future, these genetically engineered mouse models will provide both insights and tools for the exploitation of RAMP-based therapies for the treatment of human diseases.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

The family of mammalian receptor activity modifying proteins (RAMPs) offers an exciting opportunity to elucidate the pharmacological and biological complexities of G protein-coupled receptor (GPCR) signaling while also enabling the unique pharmacological manipulation of numerous GPCRs that are involved in a wide variety of physiological process and disease conditions. The wide tissue distribution of RAMP proteins and their evolutionary conservation suggests that they have much broader functions than just mediating the ligand binding specificity of the calcitonin receptor-like receptor, through which the RAMPs were originally identified by Foord and colleagues.[1] In fact, numerous studies by several groups have demonstrated that RAMPs can functionally interact with at least 5 other receptors of the Secretin Family,[2] the calcium sensing receptor[3] as well as the nonreceptor cytoskeletal protein, alpha tubulin.[4] Moreover, pharmacological and biochemical studies in cultured cell lines suggest that RAMPs can modify numerous aspects of GPCR signaling, including ligand binding, receptor desensitization, receptor trafficking and second messenger signaling and so they make attractive pharmacological targets.[5]

These exciting and seemingly expanding functions for RAMP proteins also complicate our efforts to better understand the physiological significance of RAMPs in normal and disease conditions. Therefore, our laboratory has employed a gene targeting approach to generate mouse models with absent and/or reduced expression of each RAMP and then comparatively phenotype the models to uncover the most pertinent physiological functions of the RAMPs. Several other groups have also independently generated individual RAMP knockout mice so that the comparative evaluation of different mouse lines, genetic backgrounds and phenotypes can be extremely valuable. Finally, the in vivo over-expression of RAMP proteins in specific cell types using conventional transgenic approaches has also been utilized to uncover new insights into RAMP biology. Results and interpretations from these genetic animal models are summarized below.

However, we must remain cognizant of several confounding variables when trying to infer the function of RAMPs from genetic animal model phenotypes. First, if the loss of a *Ramp* gene is incompatible with life, for example with *Ramp2*, then the assessment of loss-of-function effects during adulthood is precluded. To overcome this barrier, sophisticated gene targeting approaches which can conditionally inactivate a gene either in time or in a specific tissue or cell type can be used, but this typically requires generation of additional mouse models and complex breeding schemes. Alternatively, the surviving haploinsufficient mice can be evaluated for phenotypes, but the phenotypes must be robust enough to be detected on

a heterozygous background. Secondly, because the gene expression of RAMPs is dynamically regulated in a spatio-temporal manner by a variety of stimuli and conditions, the physiological effects of loss or reduction in RAMP gene expression may not be obvious under basal conditions. Therefore, challenging the animal models so that they are under appropriate physiological conditions which mimic the spatio-temporal regulation of RAMP gene expression may be desirable. Thirdly, as we have learned from in vitro pharmacological studies, the RAMPs can interact with numerous GPCRs in a manner which is not always straightforward. For example, association of RAMPs 1, 2 and 3 with the calcitonin receptor dynamically changes the relative affinity of the receptor for the amylin ligand,[6] so that a functional knockout of one RAMP may be partially compensated for by the expression of other RAMPs with respect to amylin signaling. As another example, association of RAMPs and receptors from different species can lead to marked differences in pharmacologcial profiles,[7] so that the lessons learned from in vitro studies using combinations of reconstituted human, rat or other species receptors and RAMPs should be considered carefully when interpreting in vivo phenotypes of mouse models. Finally, as is the case with most genetically engineered mouse models, the influence of genetic background on the observed phenotype plays an important role. In our own studies, we have found drastic changes in the gene expression levels of *Ramps* between different genetic backgrounds which directly translates to a different presentation of phenotype for the disrupted allele on different genetic backgrounds.[7a]

Nevertheless, it is clear that genetically engineered animal models can provide useful and clinically-relevant insights into the broad functions of the RAMP family of proteins. As we begin to exploit RAMPs for pharmacological manipulation of GPCRs, these models, as well as those generated in the future, will provide useful in vivo tools for the preclinical testing of relevant compounds.

## RAMP1

### Gene Targeted Deletion of RAMP1

The CLR-RAMP1 heterodimer makes a functional receptor for CGRP, a neuropeptide which plays important roles in the regulation of cardiovascular and immune systems. A mouse line lacking the *Ramp1* gene ubiquitously was generated utilizing the Cre-loxP strategy.[8] Although *Ramp1*$^{-/-}$ mice had no obvious abnormalities in their appearance, they had slightly elevated basal blood pressure with normal heart rate compared to wildtype mice, as measured by carotid catheters under anesthesia. Experiments measuring the

activity of the vasodilators αCGRP, acetylcholine and sodium nitroprusside were performed in *Ramp1*$^{-/-}$ and wildtype mice to address the function of RAMP1 in mediating vasodilation. *Ramp1*$^{-/-}$ and wildtype mice exhibited similar responses to acetylcholine and sodium nitroprusside, but *Ramp1*$^{-/-}$ mice failed to respond to the vasodilatory effects of αCGRP. These data demonstrate that the lack of a response to αCGRP in *Ramp1*$^{-/-}$ mice is not due to any abnormalities in the vascular smooth muscle cells or endothelial cells and confirm that the vasodilatory action of αCGRP is dependent on the availability of CLR-RAMP1 receptor complex. Interestingly, *Ramp1*$^{-/-}$ mice had elevated levels of serum CGRP, which further confirms that in spite of the availability of the ligand, the lack of the functional receptor leads to dysregulation of vasodilation.

Although CLR-RAMP1 receptor complex is defined as a CGRP receptor, little is known about the differential effects of the two isoforms of CGRP, αCGRP and βCGRP, on this receptor. Responses to αCGRP and βCGRP on the relaxation of aortic rings from *Ramp1*$^{-/-}$ and wildtype mice demonstrated that CLR-RAMP1 serves as a receptor for both isoforms, but that the α-isoform elicits a stronger effect than the β- isoform of CGRP. In support of the promiscuous nature of RAMP-receptor pharmacology, differential responses to relaxation of the aortic rings to adrenomedullin in *Ramp1*$^{-/-}$ and wildtype mice suggested that adrenomedullin may partially transduce signaling via CLR-RAMP1 receptor.

Administration of lipopolysaccharide (LPS) in *Ramp1*$^{-/-}$ and wildtype mice helped to elucidate an important function for CGRP in regulating inflammation.[8] Interestingly, LPS-induced cytokine production and inflammation caused a remarkable increase in serum CGRP levels of *Ramp1*$^{-/-}$ mice compared to wildtype mice. These data suggest a mechanism where CGRP, via the CLR-RAMP1 receptor, carries out an anti-inflammatory role by suppressing the production of proinflammatory cytokines.

Altogether, findings from the characterization of *Ramp1*$^{-/-}$ mice have confirmed the crucial role of RAMP1 in the CGRP signaling pathway, particularly in the cardiovascular and inflammatory processes.

## Transgenic Overexpression of RAMP1

A transgenic mouse line that expresses *hRAMP1* primarily in neurons and glia has been generated by Zhang et al.[9] Nestin/hRAMP1 mice express *hRAMP1* RNA in the brain, trigeminal ganglion, spinal cord and dorsal root ganglion. Quantitative gene expression showed that the mRNA levels of *hRAMP1* in the brain were 50% of the endogenous mouse *Ramp1*

expressed in neuronal tissues. Therefore, the overall increase in *RAMP1* mRNA expression is modest, but importantly not supra-physiological, in the brain and the trigeminal ganglion of nestin/hRAMP1 transgenic mice. As a consequence, increased production of hRAMP1 in the trigeminal ganglia enhanced CGRP-induced release of substance P from these neurons, leading to plasma extravasation and inflammation in subcutaneous tissues (such as paws and whisker pads). The effect of CGRP-triggered neurogenic inflammation could be blocked by the CGRP antagonist, CGRP8-37; further indicating that trigeminal RAMP1 is involved in CGRP-induced inflammation. Importantly, the expression of *hRAMP1* mRNA exclusively in neuronal tissues, but not in subcutaneous tissues, confirms the involvement of trigeminal hRAMP1 in CGRP-evoked inflammation. Therefore, the finding that the availability of RAMP1 is rate-limiting for the actions of CGRP in the trigeminal ganglion opens a new dimension on understanding trigeminal pathologies, such as migraine, by the regulation of CGRP and its receptor, CLR/RAMP1.

More recently, Chrissobolis et al characterized the protective effects of RAMP1 in the vasculature using a transgenic mouse that ubiquitously expresses *hRAMP1*[10]. Quantitative PCR in several tissues showed ubiquitous expression of *hRAMP1* in these transgenic mice. The transgene did not affect the endogenous levels of mouse *RAMP1* because the gene expression levels were not different when compared to the controls. In vitro studies involving carotid and basilar arteries of the transgenic mice exhibited a robust response to CGRP-mediated vasodilation, when compared to other vasodilatory agents such as adrenomedullin or acetylecholine, confirming the selective response of the hRAMP1 rich endothelium to CGRP. Additionally, in vivo studies exhibited vasodilation of the cerebral arteries in a CGRP-specific manner in *hRAMP1* transgenic mice compared to controls. In the same transgenic *hRAMP1* mice, Sabharwal et al[11] have shown that these mice display an attenuated response to Ang II-induced hypertension, suggesting that increased expression of RAMP1 is vasoprotective. More interestingly, when the carotid arteries of mice were treated with acetylcholine in the presence or absence of Ang II to test AngII-mediated vascular dysfunction, hRAMP1 expression in transgenic mice abrogated the effects of Ang II on the vasculature. This is a novel finding attributing the functional role of RAMP1 in Ang II mediated vascular dysfunction. Consistent with studies by Zhang et al, this particular study also showed that increased expression of RAMP1 displays selective and enhanced vascular response to CGRP but not adrenomedullin, thereby making the effect of CGRP RAMP1-limited.

## RAMP2

### Gene Targeted Deletion of RAMP2

Unlike *Ramp1* and *Ramp3* null mouse models which survive to adulthood, *Ramp2*$^{-/-}$ mice are embryonic lethal at mid gestation.[12-14] These findings demonstrate that the endogenous expression of *Ramp1* and *Ramp3* are unable to compensate for the loss-of-function of *Ramp2* in vivo. Amazingly, comparative phenotyping on similar isogenic genetic backgrounds revealed that gene knockout mice for AM,[15] Calcrl[16] and RAMP2[13,14] share a conserved phenotype consisting of mid-gestation embryonic lethality characterized by generalized edema. The conservation of phenotypes between the AM, Calcrl and Ramp2 knockout lines not only highlights the importance of AM signaling for embryonic survival but also provides the first genetic evidence to substantiate the RAMP-GPCR signaling paradigm, and specifically the function of the CLR-RAMP2 complex, in vivo.

Generalized edema has been reported in other knockout mice that encode for genes crucial for lymphangiogenesis.[17] Characterization of *AM*$^{-/-}$, *Calcrl*$^{-/-}$ and *Ramp2*$^{-/-}$ mice, which were all generated and maintained on an isogenic 129/S6-SvEv-TC1 background, revealed that the principle cause of the edema was due to defects in lymphatic vascular development.[13] The jugular lymph sacs of the *Ramp2*$^{-/-}$ mice were significantly smaller than those of their control littermates. In vivo BrdU incorporation assays further demonstrated a reduced rate of lymphatic endothelial cell proliferation compared to blood endothelial cells in all mutant lines tested. Electron microscopy studies showed that the junctional barrier of blood and lymphatic vessels remained intact, but that the lymphatic endothelial cells appeared thin and often necrotic in the *Ramp2*$^{-/-}$ mice. In vitro studies showed that AM signaling, mediated through RAMP2-CLR receptors, causes an enhanced activation of the MAPK/ERK signaling cascade, which is essential for endothelial cell survival and driving normal developmental lymphangiogenesis. Because these studies, and findings from other groups,[18-20] show that the expression of the *Calcrl* and *Ramp2* genes is regulated by the lymphatic-specific transcription factor, Prox1,[18,21] their expression is preferentially higher in lymphatic endothelial cells compared to blood endothelial cells. Other cardiovascular defects in the Ramp2$^{-/-}$ embryos, which are also present in the AM[15] and Calcrl[16] null models, include thin vascular smooth muscle walls and small hearts with thin compact zones and disorganized ventricular trabeculae. Together, these data identify a previously unrecognized role for RAMP2-mediated AM signaling in the development and function of the cardiovascular system and highlight the importance of CLR-RAMP2 signaling as a pharmacologically-tractable regulator of lymphatic proliferation.

Ichikawa-Shindo et al have also reported an independent line of Ramp2 null embryos which were generated by global CAG-Cre driven excision of a floxed *Ramp2* allele.[12] These animals also demonstrated extensive generalized edema and pericardial effusion. Ultrastructural analysis revealed defects in blood endothelial and vascular smooth muscle structure resulting in the presence of occasional hemorrhagic plaques. Using RNA lysates isolated from whole embryo extracts, significant reductions in the expression of endothelial adhesive genes was shown in *Ramp2*$^{-/-}$ mice compared to wildtype controls, suggesting that the expression of *Ramp2* is required for maintaining the blood vessel barrier. The subtle phenotypic differences between the two independent *Ramp2* null mouse strains could be influenced by the different genetic backgrounds. Importantly, the lymphatic and blood vascular defects are not mutually exclusive and actually shed greater insights into the complexity of and interplay between the blood and lymphatic vascular systems in maintaining tissue fluid balance.[22]

## Haploinsufficiency for RAMP2

The embryonic lethality of *Ramp2* global knockout mice precludes the study of RAMP2 loss-of-function in adult animals, but heterozygote animals expressing half the normal levels of *Ramp2* have been useful to study. *Ramp2* heterozygous females on an SvEv129/S6 genetic background have severely reduced fertility with litter sizes approximately one-third of wildtype mice and other isogenic RAMP models.[14] While reduced fertility is also a hallmark feature of the *AM*$^{+/-}$ female mice,[23-24] the fertility defects of the *Ramp2*$^{+/-}$ females is much more prominent and severe, and in fact contributes to difficulties in maintaining the strain. Our most recent studies suggest that the fertility defects can be attributed to marked endocrine imbalances in the hypothalamic-pituitary axis which are not observed in the *AM*$^{+/-}$ model (M. Kadmiel and K. Fritz-Six, unpublished observations). Therefore, a divergence in phenotypes between the *Ramp2*$^{+/-}$ and *AM*$^{+/-}$ mice (all maintained on an identical genetic background), suggests that RAMP2 may have broader in vivo roles beyond its requirement for generating an AM receptor with CLR. Consistent with our previous findings, a modest genetic reduction in *Ramp2* had no affect on basal blood pressures or heart rates of conscious male or female mice, as measured by the tail cuff method.[14]

The *Ramp2* heterozygote mice reported by Ichikawa-Shindo and colleagues also survived to adulthood, but unlike the Dackor et al *Ramp2*$^{+/-}$ mice these animals showed modest increases in basal systolic blood pressure, as measured in anesthetized animals using carotid artery catheters.[12] Consistent with the canonical paradigm of RAMPs regulating CLR's ligand binding specificity, the *Ramp2*$^{+/-}$ mice showed a markedly reduced vasodilatory response to AM treatment, but not to calcitonin gene related peptide (CGRP). In a series

of elegant in vivo angiogenesis assays, the *Ramp2*$^{+/-}$ mice also revealed a reduced angiogenic response to VEGF, decreased neovascularization and increased in vivo vascular permeability in the footpad, skin and brain. These studies, which are consistent with the discoveries made in the global *Ramp2* knockout embryos, highlight the importance of RAMP2-mediated signaling in regulating pathological angiogenesis and vascular permeability.

### Transgenic Overexpression of RAMP2

The effects of overexpression of RAMP2 in vivo have been investigated using a transgenic approach in which *Ramp2* was overexpressed in smooth muscle, under the control of an α-actin promoter.[25] Consistent with a modest role for RAMP2 in regulating basal blood pressures, the *Ramp2* transgenic mice had mean arterial blood pressures and heart rates that were indistinguishable from their wildtype littermates. As expected, the *Ramp2* transgenic mice exhibited potent and selective responsiveness to vasodilatory peptides. For example, while AM treatment of *Ramp2* transgenic mice enhanced vasodilation leading to increased stroke volume and reduced end-systolic pressure compared to similarly treated wildtype animals, the administration of CGRP did not result in appreciable differences between the *Ramp2* transgenic mice and wildtype animals. These data support a principal physiological function of RAMP2 in mediating the vasodilatory effects of AM in vascular smooth muscle cells. The *Ramp2* transgenic animals also showed increased inflammatory fluid extravasation after subcutaneous injection of substance P with cotreatment of AM, but not with CGRP cotreatment, again supporting an important role for CLR-RAMP2 mediated regulation of tissue fluid balance.

## RAMP3

### Gene Targeted Deletion of Ramp3

*Ramp3* null mice survive to adulthood without any obvious developmental problems.[14] Basal blood pressures and heart rates are unaffected by the loss of *Ramp3*, and both male and female mice reproduce normally compared to their wildtype littermates. Despite the fact that global *Ramp3* null mice exhibit normal food and water intake, they suffer from markedly reduced body weights after approximately 6 months of age. Although the mechanisms underlying this phenotype are not yet understood, the age-dependant lean phenotype did not affect health or longevity up to 18 months of age.

### Lessons Learned from Comparative Phenotyping

A summary of the most well-characterized phenotypes discovered in genetic RAMP mouse models is provided in Table 1. Characterization of any individual RAMP mouse model reveals important information about RAMP biological functions in vivo. Comparing agonist activity in RAMP mice has provided direct in vivo evidence that RAMPs covey specificity for different ligands, such as AM and CGRP under physiological conditions. For example, AM treatment, but not CGRP, shows potent effects on vasodilation and inflammation in RAMP2 animals, while CGRP treatment, but not AM, reveals physiological effects in Ramp1 transgenic mice.

A comparative approach to phenotyping between models can also provide powerful information, as long as the comparisons are performed on similar or identical genetic backgrounds. Conservation of phenotypes between genetic RAMP models and genetic models of their putative ligands (for example, the conserved phenotypes of *AM*, *Calcrl* and *Ramp2* mice) can reveal physiologically important signaling paradigms that can be exploited for disease treatment or therapies. On the other hand, highly divergent or unexpected phenotypes in the genetic RAMP models can reveal previously unrecognized roles for RAMPs in mediating the signaling of other ligands and receptors.

It is also this direct comparison of RAMP models that allows us to better understand compensatory effects of the RAMPs for one another. For example, embryonic lethality of the *Ramp2* null mice demonstrates that loss of RAMP2 can not be compensated for by other RAMP family members, either because they are not expressed in the appropriate place and/or time or because RAMP1 and RAMP3 have nonredundant functions with RAMP2. In contrast, global loss of either RAMP1 or RAMP3 does not affect survival, perhaps because the expression of other RAMPs compensates for their absence. More definitive answers to these compensatory paradigms can come from careful evaluation of homeostatic responses in gene expression and protein expression of RAMPs in specific cells and tissues,[14] but we are currently hindered by the lack of effective, commercially available murine antibodies for RAMP proteins.

## CONCLUSION AND FUTURE DEVELOPMENTS

Much can be gained from directly comparing phenotypes between gain-of-function and loss-of-function alleles for each RAMP model. The prediction is that altering the genetic dosage of a RAMP will result in a range

**Table 1**. Comparative phenotyping of RAMP mouse models

| | *RAMP1 –/–*[8] | *RAMP1 TG*[9,10] | *RAMP2 –/–*[12-14] | *RAMP2 +/–*[12-14] | *RAMP2 TG*[25] | *RAMP3 –/–*[14] |
|---|---|---|---|---|---|---|
| Survive to Adulthood | Yes | Yes | No | Yes | Yes | Yes |
| Altered Blood Pressure | Yes | N/A | N/A | Yes/No | Yes | No |
| CGRP Relaxation Response | Reduced | Enhanced[10] | N/A | Normal | Normal | N/A |
| AM Relaxation Response | Reduced | Normal[10] | N/A | Reduced | Enhanced | N/A |
| Infammation/Fluid Extravasation basally # | Enhanced (LPS) | N/A | N/A | Enhanced (histamine) | Normal (Substance P) | N/A |
| Infammation/Fluid Extravasation with CGRP Tx | N/A | Enhanced[9] | N/A | N/A | Reduced* | N/A |
| Infammation/Fluid Extravasation with AM Tx | N/A | N/A | N/A | N/A | Enhanced* | N/A |
| Fertility Defects | No | No | N/A | Yes/No | No | No |
| Body Weight Defects | No | No | N/A | No | No | Yes |

# Inflammatory treatment indicated in parentheses
*coinjected with Substance P

of phenotypes. In fact, studies on the angiogenic and permeability effects of RAMP2 in both transgenic and gene targeted models provides an elegant example of the strength of this comparative phenotyping approach. In the future, additional animal models which expand our repertoire of both spatial and temporal manipulation of RAMP gene expression will continue to shed new insights in the physiological functions of RAMP proteins in normal and pathological conditions and potentially elucidate processes in which the pharmacological manipulation of RAMPs may be beneficial for treating human disease.

## ACKNOWLEDGEMENTS

The authors wish to acknowledge the support from the National Institutes of Health grants HL091973 and HD060860 and The Burroughs Wellcome Fund to KMC and The American Heart Association Established Investigator Award to KMC and Pre-doctoral Fellowship to MK.

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CHAPTER 6

# RAMPs AS DRUG TARGETS

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**Abstract:** The receptor activity-modifying protein (RAMP) family of membrane proteins regulates G protein-coupled receptor (GPCR) function in several ways. RAMPs can alter their pharmacology and signalling as well as the trafficking of these receptors to and from the cell surface. Accordingly, RAMPs may be exploited as drug targets, offering new opportunities for regulating the function of therapeutically relevant RAMP-interacting GPCRs. For example, several small molecule antagonists of RAMP1/ calcitonin receptor-like receptor complexes, which block the actions of the neuropeptide calcitonin gene-related peptide are in development for the treatment of migraine headache.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

G protein-coupled receptors (GPCRs) are the largest single grouping of proteins which are considered "druggable". GPCRs represent the major signalling system in mammalian cells and are subdivided into three groups: Family A, the largest family, containing receptors for prototypical neurotransmitters and hormones; Family B, incorporating receptors for peptide hormones such as calcitonin (CT) and secretin; and Family C, which includes receptors for small molecules such as glutamate and GABA.[1] GPCRs, like other signalling proteins, can form oligomeric protein arrays which may be critical to many aspects of their function. For many GPCRs it is likely that constitutive dimers or oligomers act as the core functional unit. Furthermore, these receptors, as either a monomer or oligomeric complex, may also interact with other proteins that regulate their function. Interactions such as these create novel opportunities for drug discovery and development as exemplified by the receptor activity-modifying protein (RAMP) family. This chapter will initially consider the range of GPCRs which can interact with RAMPs. The consequences of these interactions will then be reviewed, to indicate the range of functions that could be modulated by targeting RAMPs. Finally, mechanisms for directing drugs towards RAMPs and their complexes will be considered.

## RAMPs AND THEIR INTERACTIONS WITH RECEPTORS

As comprehensively described elsewhere in this book, RAMPs are a family of 3 proteins initially shown to regulate the glycosylation, transport and pharmacology of the CT receptor-like (CL) receptor (CLR).[2,3] RAMP1/CLR constitutes a CT gene-related peptide (CGRP) receptor, whereas RAMP2/CLR and RAMP3/CLR exhibit distinct adrenomedullin (AM) receptor phenotypes.[4-7] RAMPs revealed a novel mechanism for producing diversity in receptor response. RAMPs were also shown to interact with the related CT receptor to create amylin receptors, with each RAMP/CT receptor complex displaying a distinct phenotype.[8,9]

CLR and the CT receptor are Family B peptide hormone receptors; subsequent analysis of other members of this receptor family for potential interactions with RAMPs, revealed additional RAMP-receptor partners. There were different degrees of specificity in these interactions. The VPAC1 receptor interacted with all 3 RAMPs, like the CT receptor and CLR.[3] However, the PTH1 and glucagon receptors interacted specifically with RAMP2, and the PTH2 receptor specifically with RAMP3. There was no measurable translocation of RAMPs with the VPAC2, GHRH, GLP-1 or GLP-2 receptors.[3] There is some evidence that the VPAC1/RAMP2 interaction

may alter the signalling of this receptor (see below) but otherwise little is known of the consequences of RAMPs on the function of these receptors.

A chaperone role for RAMPs 1 and 3 (not RAMP2), with the Family C, calcium sensing receptor (CaS receptor) trafficking has also been reported.[10] This illustrates the potential for RAMP modulation of receptors outside of Family B GPCRs. The broad distribution of RAMPs throughout cells and organs of the body, beyond the localisation of known receptor partners (reviewed in refs. 6,11,12), provides significant scope for additional receptor partners to RAMPs to be identified. This potential to interact with and modulate the function of many GPCRs offers access to novel strategies for drug development.

## THE CONSEQUENCES OF RAMP-RECEPTOR REGULATION

### RAMP-Regulation of GPCR Pharmacology

RAMPs are the critical machinery that drives the pharmacology of the CT peptide family.[7] This family comprises CT, amylin, CGRP, AM and AM2 (also known as intermedin).[13,14] Some CT peptide family receptors are validated pharmaceutical targets for diseases including diabetes, migraine (see below) and osteoporosis. There are two Family B GPCRs for these peptides, the CT receptor and the CLR. CLR by itself is only poorly-expressed at the cell surface and will bind no known endogenous ligand. However, when paired with RAMP1, this complex is the CGRP receptor. In contrast, two pharmacologically-distinct AM receptor subtypes are formed from RAMP2/CLR ($AM_1$ receptor) or RAMP3/CLR ($AM_2$ receptor) complexes. It has been reported that the $AM_2$ receptor also recognises CGRP with reasonable affinity, depending on the species from which receptor subunits are derived.[5] Similarly, the CGRP receptor has moderately high affinity for AM.[7]

The CT receptor without RAMP appears to be a conventional Family B GPCR and is sufficient for CT binding with no apparent requirement of RAMPs for cell surface expression of this receptor.[7] In the absence of RAMP, the CT receptor has low affinity for amylin but the CT receptor expressed with RAMP1, 2 or 3 respectively, gives three subtypes of high-affinity amylin ($AMY_{1-3}$) receptor. Several CT receptor splice variants have been reported; their interaction with RAMPs generates a large number of possible amylin receptor subtypes.[6] There is considerable complexity in this system with RAMPs enabling substantial pharmacological diversity from only two GPCR genes.

No other RAMP pairings with GPCRs have yet been shown to cause changes in pharmacological phenotype but there could be novel combinations

which may reveal that this ability of RAMPs to modify pharmacology is more widespread than is currently understood.

There are two likely mechanisms by which RAMPs could influence GPCR pharmacology. A direct contribution of binding epitopes to the relevant ligands is possible or they could act indirectly by altering the conformation of the GPCR. There is strong experimental support for interactions between the long N-termini of the RAMP and the CT receptor or CLR determining the specific pharmacology of each receptor complex.[4,6,15-20] The interface between these proteins is likely to generate the unique structures required for the binding of selective ligands. Amino acid 74 in RAMP1 and RAMP3 has a particularly influential role in determining AM and small molecule antagonist affinity (see below).

## RAMP-Regulated Receptor Signalling

There is evidence that RAMPs may also modify the signalling profile of receptors. Most information is available for RAMP/CT receptor-derived AMY receptors. In early studies, receptor isoform- and cell background-dependent differences in the ability of RAMP2 to create a high affinity AMY phenotype were identified. For example, in COS-7, and rabbit aortic endothelial cells, RAMP2 only weakly engenders amylin binding from the $CT_{(a)}$ receptor isoform, in contrast to strong induction of AMY phenotype when co-expressed with the $CT_{(b)}$ receptor isoform.[8,9,21] The presence of the 16 amino acid insert in the first intracellular domain of the $CT_{(b)}$ receptor results in reduced Gq-mediated signalling and receptor internalisation.[22] The situation is different in CHO-P cells where co-expression of either receptor isoform with RAMP2 led to strong induction of AMY phenotype.[21] This led to the hypothesis that a component of the cellular background, potentially G proteins, could modulate RAMP/CT receptor interaction. CGRP potency for cAMP formation at RAMP/CT receptor complexes tracked with the C-terminal RAMP region as demonstrated using chimeras of RAMP1 and RAMP2 where the intracellular C-terminus was exchanged. In contrast, the binding affinity tracked with the RAMP N-terminal domain, indicating that the RAMP C-terminus played a direct role in signalling.[16] RAMP C-terminal truncation also decreased amylin binding, an effect that could be partially reversed by overexpression of $G\alpha s$,[23] suggesting that the RAMP C-terminus played a direct role in G protein coupling. Further investigation revealed a preferential coupling of $AMY_1$ and $AMY_3$ receptors to Gs *versus* Gq, relative to $CT_{(a)}$ receptors expressed alone. Furthermore, $G\alpha$ subtypes differentially influence the amount of $^{125}$I-amylin binding to $RAMP/CT_{(a)}$ complexes; Gs over-expression increased binding of $RAMP2/CT_{(a)}$ and $RAMP3/CT_{(a)}$ complexes but Gq only increased binding at $RAMP3/CT_{(a)}$ complexes.[24] Taken together, these data support a direct role for the RAMP C-terminal

domain in the G protein interaction profile of AMY receptors, potentially in an individual RAMP-dependent manner.

In contrast to this action at RAMP/CT receptor complexes, RAMPs do not appear to be directly involved in G protein coupling of RAMP/CLR complexes.[23] These complexes interact with CGRP receptor-component protein (RCP) which plays an important role in CGRP and AM receptor signalling.[25] This intracellular peripheral membrane protein is crucial for Gs-mediated cAMP signalling at CGRP and AM receptors and may be an alternative mechanism for regulating signalling at these complexes.[25] To date, there is no evidence for a role for RCP in signaling of other RAMP/GPCR complexes.

As outlined above, the VPAC1 receptor is also a RAMP-interacting receptor. Modulation of receptor signalling by RAMPs has also been observed at this receptor. Here, RAMP2 over-expression caused a selective augmentation of phosphoinositide hydrolysis (presumably downstream of Gq) in the absence of an alteration in cAMP formation.[3] It has been speculated, however, that this effect may be explained by changes in compartmentalisation of RAMP2/VPAC1 receptor complexes rather than a direct effect on G protein coupling. Although RAMPs have now been demonstrated to interact with several receptor partners, the effect on the signalling profile of these receptors has not been investigated. Nonetheless, these studies clearly indicate the potential for selective modulation of receptor signalling through targeting of the RAMP/receptor interface.

## RAMP-Regulated Receptor Trafficking

A prototypical function of RAMPs is to act as a chaperone for CLR and promote its cell-surface expression.[2] RAMPs associate with this receptor in the endoplasmic reticulum and promote terminal glycosylation in the Golgi. There appears to be a similar mechanism of action for CaS receptor trafficking to the cell surface by RAMPs 1 or 3 (but not RAMP2). These RAMPs deliver the receptor from the endoplasmic reticulum to the Golgi, thereby promoting glycosylation.[10] Whether RAMPs also impact upon the expression of other receptors is yet to be investigated.

RAMPs are also in a position to influence the extent and fate of internalisation of receptors. RAMPs have been shown to modulate receptor trafficking during down regulation, where the RAMP/CLR complex is internalised with β-arrestin.[26] RAMP2-containing $AM_1$ receptors have been reported to show greater internalisation than RAMP3-containing $AM_2$ receptors when expressed in HEK293 cells, although the responsible mechanism is unclear.[27] There is a PDZ-like domain in the C-terminus of RAMP3 which can interact with the N-ethylmaleimide-sensitive factor (NSF). Under the appropriate circumstances this interaction can lead to recycling

of the RAMP3-containing $AM_2$ receptor after internalization; by contrast, in the model cells used in these experiments, there was no recycling of $AM_1$ or CGRP receptors.[28] Thus RAMP-interacting drugs have the potential to influence receptor trafficking.

## Consequences of Changes in RAMP Expression/Activity

In consideration of RAMPs as drugs targets, how RAMP expression may change drug action and how drugs may change RAMP expression are both of relevance. There is clear evidence for regulation of RAMP expression: in disease, in response to drugs/hormones and physiologically which in turn may affect drug action.[29,30] Many of these studies, for example those describing RAMP regulation in heart failure, hypertension and renal failure have been reviewed.[11] New insight into the function of RAMPs can now be gleaned from knockout mouse models for each RAMP gene. For example, although both RAMP2 and RAMP3 generate similar AM receptors in association with CLR, RAMP2 and RAMP3 knockout mice have revealed distinct roles for these two proteins.[31] Deletion of the RAMP2 gene results in a lethal phenotype, likely resulting from abnormalities in the blood and lymphatic vasculature.[32,33] On the other hand, RAMP3 knockout mice are viable, with no obvious phenotype until old age when they are lighter than their wild-type counterparts.[31] The phenotype is different for mice in which RAMP1 expression is disrupted; these exhibit hypertension and a dysregulated immune response.[34]

A mouse model with neural over-expression of human RAMP1 has indicated that this protein might be functionally limiting for the neuropeptide CGRP. From the perspective of drug discovery, this is highly relevant as alterations in the expression of RAMP could have profound consequences on responsiveness to endogenous ligands or drugs that act at RAMP-receptor complexes. Mice with ~2-fold increase in RAMP1 mRNA in the brain/trigeminal ganglia were sensitized to CGRP-induced plasma extravasation, a measure of neurogenic inflammation.[35] A second example of the consequences of changes in RAMP expression was shown in a mouse model with over-expression of RAMP2, primarily in smooth muscle. Here, the mice were sensitized to the vasodilatory actions of AM; the mice displayed enhancement of AM-induced decreases in blood pressure.[36] The concept of RAMP expression as a limiting factor for receptor function has implications for the onset and progression of disease and also its treatment.

Although the impact of RAMP expression levels has been discussed in the context of the CT family peptides and receptors as they are the best characterised, the potentially broader roles of RAMPs also needs to be

considered. Importantly, caution should be applied in interpreting RAMP protein expression data due to the poor quality of antibodies that are often used in these studies. Whilst there are commercially available RAMP antibodies, the authors' (unpublished observations) and others have noted issues with the specificity of these antibodies.[37]

## MECHANISMS OF TARGETING RAMPs BY DRUGS

### Overview

Theoretically, RAMPs could be targeted directly by drugs or the complex between the RAMP/receptor complex could be exploited. A drug acting at a RAMP/receptor complex could interact with both the RAMP and the receptor (as with BIBN4096BS/MK0974, see below). However, in principle, if the RAMP substantially altered the structure of the receptor with which it interacted, then this novel structure by itself might be targetable by selective drugs. The extracellular portions of the RAMP/receptor complex are likely to receive the most attention for drug development; however, drugs that bound to the C-terminus of the RAMP might usefully alter receptor trafficking or signalling profiles (see above). Though challenging to produce, agents that prevented RAMP-receptor association in the endoplasmic reticulum could prevent the appearance of such complexes at the cell surface.

There is the potential for interactions between RAMPs and receptors to also be allosterically promoted to enhance cell-surface expression. This may be particularly relevant for naturally-occurring, disease-causing mutations of GPCRs, where defective cell-surface expression is an important contributor to receptor dysfunction (e.g., the CaS receptor).[38] Since the effects of allosteric modulators may depend on the nature of the specific protein-protein interaction that is being modified,[39] there is scope for such drugs to be specific for individual RAMP/GPCR complexes.

At this stage, options for structure-based drug design against RAMPs are still limited due to incomplete structural data. However, the crystal structure of the extracellular N-terminus of human RAMP1 alone revealed 3 $\alpha$-helical domains and confirmed the presence of 3 disulphide bonds between cysteines 1-5, 2-4 and 3-6.[40] This structure validated a previously predicted disulphide bonding pattern and *ab initio* model for RAMP1.[41] Recently, the structure of the extracellular N-terminus of RAMP1, together with that of CLR was reported and will undoubtedly open doors to other structures and drug design; this structure is discussed in more detail below.[42]

## Direct Targeting of RAMPs

RAMPs could be targeted directly, either by traditional (if currently hypothetical) conventional small molecule inhibitors, by developing RNAi therapeutics or antibodies which interact directly with RAMPs. However, as RAMPs are present at the cell surface as part of a receptor complex, it may be challenging to find suitable epitopes that could be targeted with antibody-based therapies. This may explain why it has been so difficult to generate antibodies that are suitable as experimental tools (see above).

Broad disruption of RAMP interactions may have unforeseen consequences due to the widespread distribution of these proteins and our limited understanding of the potential breadth of RAMP function. Nonetheless, RAMP-targeted drugs, if they can be developed, would provide great utility in understanding RAMP function and could be empirically examined for therapeutic potential.

## Targeting the RAMP-Receptor Complex

While the future of therapeutics that target the RAMP itself is unclear, there clear evidence that RAMP/receptor complexes can be successfully targeted. This is illustrated by the development of CGRP antagonists (e.g., BIBN4096BS (Olcegepant) and MK0974 (Telcagepant)).[6,43-47] These compounds have been developed for the treatment of migraine headache, which involves CGRP in its pathology. Other recent reviews should be consulted to understand the role of this peptide and the rationale for the use of CGRP antagonists in migraine treatment.[46,48] The mechanism behind the selectivity of these compounds is of most relevance to this chapter.

BIBN4096BS was the first of the nonpeptide CGRP antagonists to be developed. It has sub-nanomolar affinity for primate CGRP receptors but considerably lower affinity (~250-fold) for rodent receptors.[44,45] The exchange of a single amino acid (lysine/tryptophan) at position 74 between rat and human RAMP1 is sufficient to reverse this affinity.[15,44] The amino acid residue at this position also appears to be important for pharmacological specificity; BIBN4096BS has virtually no affinity for RAMP2 and is a weak antagonist at RAMP3-based $AM_2$ receptors; these RAMPs have glutamic acid at this position. Sensitivity to BIBN4096BS can, however, be enhanced at the $AM_2$ receptor by incorporation of the crucial tryptophan residue.[5,15,49] MK0974 also displays RAMP1-dependence, resulting in profound species and pharmacological selectivity;[43] the compounds have broadly similar chemical structures (Fig. 1). Although RAMP1 is a key determinant of the affinity of these compounds, the nature of the receptor is also important. This has been demonstrated in a comparison of BIBN4096BS affinity at RAMP1/CLR vs RAMP1/$CT_{(a)}$ complexes (~55% amino acid homology

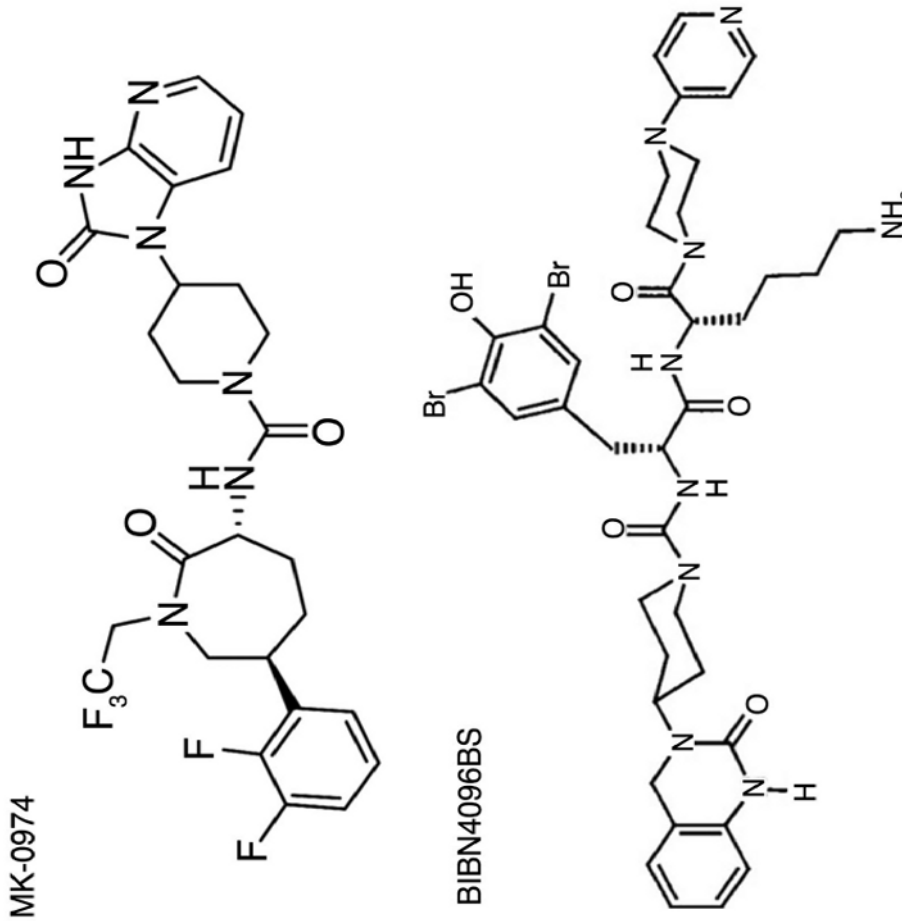


**Figure 1.** Structures of BIBN4096BS and MK0974.

in the GPCR component); BIBN4096BS was ~300-fold more effective at the CLR-based CGRP receptors.[15] Furthermore, studies using CLR/$CT_{(a)}$ chimeras indicated that the extreme N-terminus of the CLR (residues 37-63) is a crucial determinant of the affinity of BIBN4096BS-like Compounds 1 and 3.[50] Thus, the interface between these proteins was suggested to provide the binding pocket for this class of antagonist and be responsible for their selectivity. The relatively large size of BIBN4096BSBS and MK0974 by comparison with most drugs supports the notion that they need to interact with epitopes on both the RAMP and the receptor. In contrast, compounds which interact predominantly with CLR are nonselective.[50]

The recent publication of the crystal structure of the RAMP1 and CLR extracellular N-terminal domain complex with BIBN4096BS and MK0974 bound now allows us to put the earlier experimental data in perspective.[42] Specifically, both antagonists bind in a pocket formed by these two proteins, making key contacts with W74 in RAMP1, amongst other residues (Fig. 2). As expected, this contact explains the species and pharmacological selectivity of the compounds. This structure also revealed that despite the requirement of CLR for RAMP to express at the cell surface and bind CGRP, its structure appears very similar to that of other Family B GPCRs and it is likely that CGRP binds in a similar position to many other Family B GPCR peptide ligands.[51] In which case, it is easy to imagine how BIBN4096BS and MK0974 block CGRP binding. This structure clearly opens the way to designing drugs for other receptor–RAMP complexes.

Although the published structure shows a 1:1 RAMP:CLR stoichiometry, it has been proposed from biochemical studies that 2 CLR: 1 RAMP1 can exist (although the data are also consistent with 2 monomeric RAMPs binding individually to the CLR dimer).[52] It is unclear what this implies for antagonist interactions and receptor function; further work is needed.

Although discussed in the context of small molecules, drug design at RAMP/GPCR complexes need not be limited in this way. For example, at peptide receptors, the native peptides themselves could be formulated as drugs and there is considerable scope for using modified peptides. For example, Symlin (Pramlintide) is a modified form of amylin and is marketed for the treatment of diabetes.

## CONCLUSION

RAMPs provide an important opportunity for drug development. Drugs could be targeted towards RAMP-receptor complexes, likely yielding highly selective drugs or towards the RAMP, probably generating drugs with widespread effects. Recent structural advances give us useful information about how to develop such drugs with the CGRP receptor antagonists

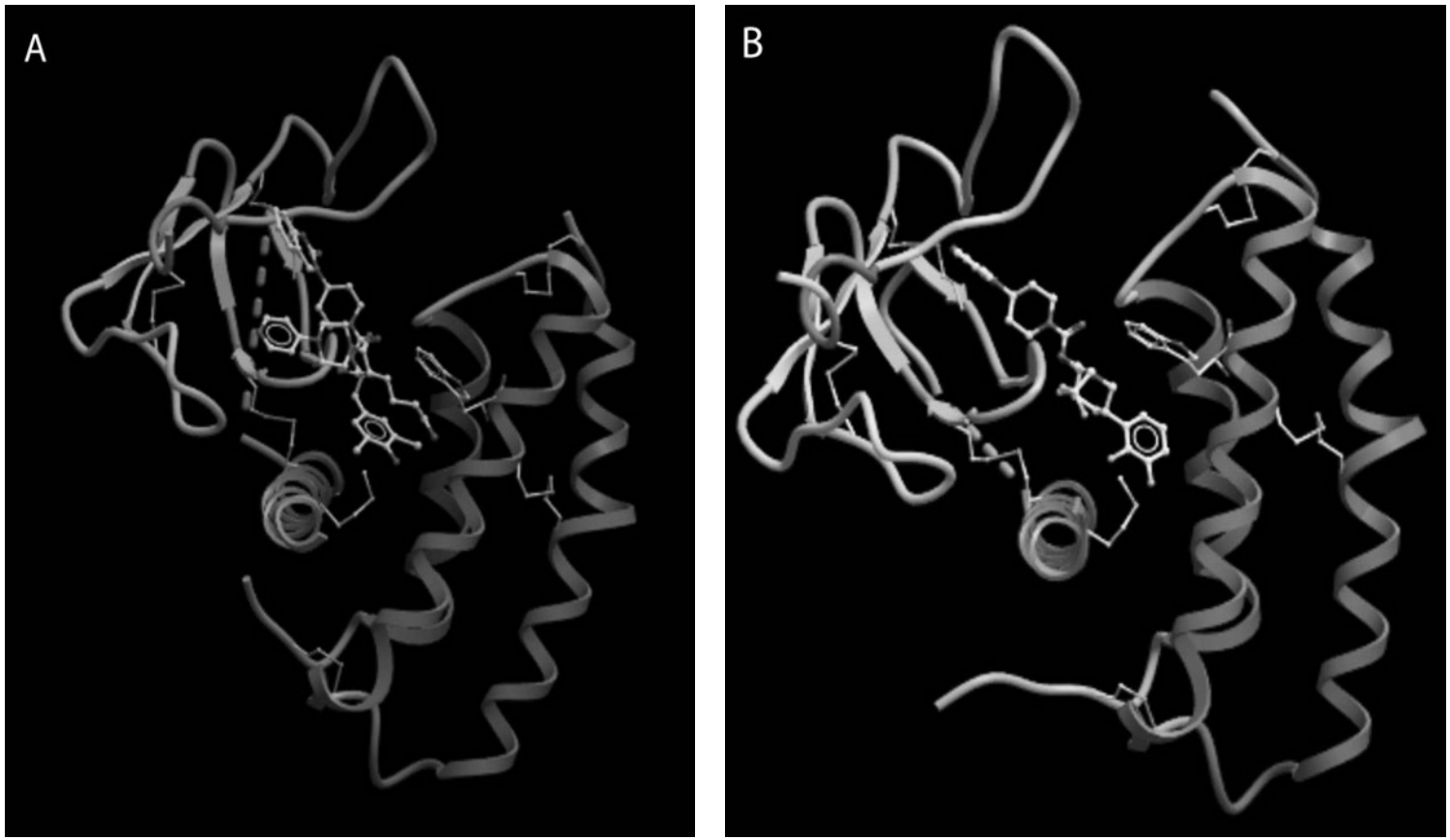


**Figure 2.** The binding of BIBN4096BS (A) and MK0974 (B) to the CLR-RAMP1 complex. In both panels, CLR is on the left and RAMP1 on the right. M42 on CLR and W74 on RAMP1, both shown by mutagenesis to be important for antagonist binding, are also shown.

providing important proof of principle that targeting the RAMP-receptor interface will yield specific, high affinity drugs. New data indicates that, in addition to receptors, RAMPs may also interact with other proteins such as tubulin.[53] Although, the physiological significance of this is unknown, it could potentially provide a mechanism for compartmentalization of RAMP-receptor complexes or novel receptor-independent functions. Furthermore, the scope for RAMP splice and sequence variants has been little explored. Further work is now needed to elucidate the structures of other RAMP-receptor complexes to exploit for drug targeting.

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CHAPTER 7

# RAMP LIKE PROTEINS
# RTP and REEP Family of Proteins

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**Abstract:** Mammalian odorant receptors (ORs) are typically retained in the endoplasmic reticulum (ER) when expressed in heterologous cells. The RTP (Receptor-Transporting Protein) and REEP (Receptor Expression Enhancing Protein) family of proteins were first identified as partners for ORs, promoting cell-surface expression and leading to functional responses in heterologous cell systems. Like RAMPs, the RTP and REEP proteins appear to partner with GPCRs to promote cell-surface expression. Unlike RAMPs, they do not appear to alter the pharmacology of the partner receptor.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

The RTP (Receptor-Transporting Protein) and REEP (Receptor Expression Enhancing Protein) family of proteins were first identified for their role in promoting cell-surface expression of odorant receptors, leading to functional responses in heterologous cell systems. Subsequent study revealed that these proteins have a more general role in partnering with GPCRs and enhancing trafficking of the receptors. In this chapter we review the roles of the RTP and REEP families in partnering with a variety of GPCRs, their implication in a variety of diseases and the possible mechanisms of action.

## RTP FAMILY

The mammalian RTP family consists of 4 members shown in Figure 1A. The RTP family proteins have a single predicted transmembrane domain located near the C-terminal end (Fig. 2A,B). Immunostaining of tagged RTP1 suggests that the N-terminal end is intracellular and the C-terminal end is extracellular, consistent with the lack of signal peptides. Co-immunoprecipitation of RTP1 with ORs indicates that RTP1 forms a complex with ORs.

A shorter form of RTP1, RTP1S, encoded from the second methionine in the same open reading frame as originally described RTP1, has a more robust effect on promotion of OR trafficking and ligand induced response of ORs.[1] The olfactory neurons predominantly express the shorter form of RTP1. However, it is not clear if translation begins at the second methionine, or if RTP1L is processed through posttranslational modification.

RTP1 and RTP2 are strongly and specifically expressed in olfactory neurons and vomeronasal neurons.[2] In contrast, RTP3 is expressed in liver, lung and testis and RTP4 is expressed in a wide variety of tissues (brain, bladder, bone marrow, colon, kidney, liver, lung, lymph node, macrophages, mammary gland, melanocytes, nasopharynx, pituitary, prostate, retinal pigment epithelium, spinal cord, spleen, testis, thymus and uterus).[3] Testicular expression of RTP3 and RTP4 is notable, as several ORs are expressed in the testis as well.[4-6] RTP1 and RTP2 both promote cell-surface expression of a wide variety of mammalian ORs. RTP3 and RTP4 have not been shown to increase cell-surface expression of any ORs, but have been shown to increase surface expression of some bitter taste receptors, the TAS2Rs, in heterologous cells.[3] In sheep, RTP4 is upregulated in response to early pregnancy, but its function is unclear.[7]

Recently, RTP4 was shown to form a complex with the μ-δ opioid receptor.[8] Although the μ-opioid receptor alone efficiently targets to the

plasma membrane in HEK293 cells, when coexpressed with the δ-opioid receptor the μ-δ receptor complex is retained in the Golgi apparatus. When co-expressed with the μ-δ opioid receptor, RTP4 binds the heterodimer, preventing retention in the Golgi apparatus and subsequent protein degradation in the proteosome. Thus, RTP4 results in an enhancement of cell-surface expression and function. These results suggest that RTP family members have a general role in forming a protein complex with GPCRs and enhancing trafficking of the receptors in the intracellular membrane structures.

## REEP FAMILY

The mammalian REEP family consists of 6 members shown in Figure 1B. REEP1 is widely expressed in different tissue types including olfactory epithelium. Some fraction of REEP1 appears to be localized on the plasma membrane when expressed heterologously.[2] However, in a separate study, subcellular fractionation and immunohistochemistry studies suggest that REEP1 localizes to mitochondria in COS7 (kidney fibroblast) and MN-1 (motor neuron) cells.[9] Mutations in REEP1 are associated with hereditary spastic paraplegia,[9-13] but neither causality nor the mechanism of action has been demonstrated. REEP1 is the only member of the REEP family whose expression is detected in the olfactory neurons, though supporting cells in the olfactory epithelium express REEP6.[2] REEP3 is a positional candidate for autism, but neither causality nor the mechanism of action has been demonstrated.[14]

The REEP1 gene contains two putative transmembrane domains (Fig. 2C). Alternatively, the hydrophobic region of first 19 amino acids may function as a signal peptide. Based on amino acid sequence, the SignalP 3.0 program[15] predicts that there is a 60% chance the N-terminus of REEP1 functions as a signal peptide. Immunostaining with the tagged protein suggests that the C-terminal end is extracellular.[2] Future studies are necessary to determine the exact topology of the REEP proteins.

## ROLE IN OLFACTION

Many GPCRs, particularly sensory GPCRs, exhibit poor surface localization in heterologous cells. Recently, a large body of evidence suggests that many of these GPCRs do not function independently, but rather are subunits of multi-protein complexes. For example, taste receptors TAS1R1, TAS1R2 and TAS1R3 show little response to taste stimuli when expressed by themselves in a heterologous system, however coexpression of T1R2 and

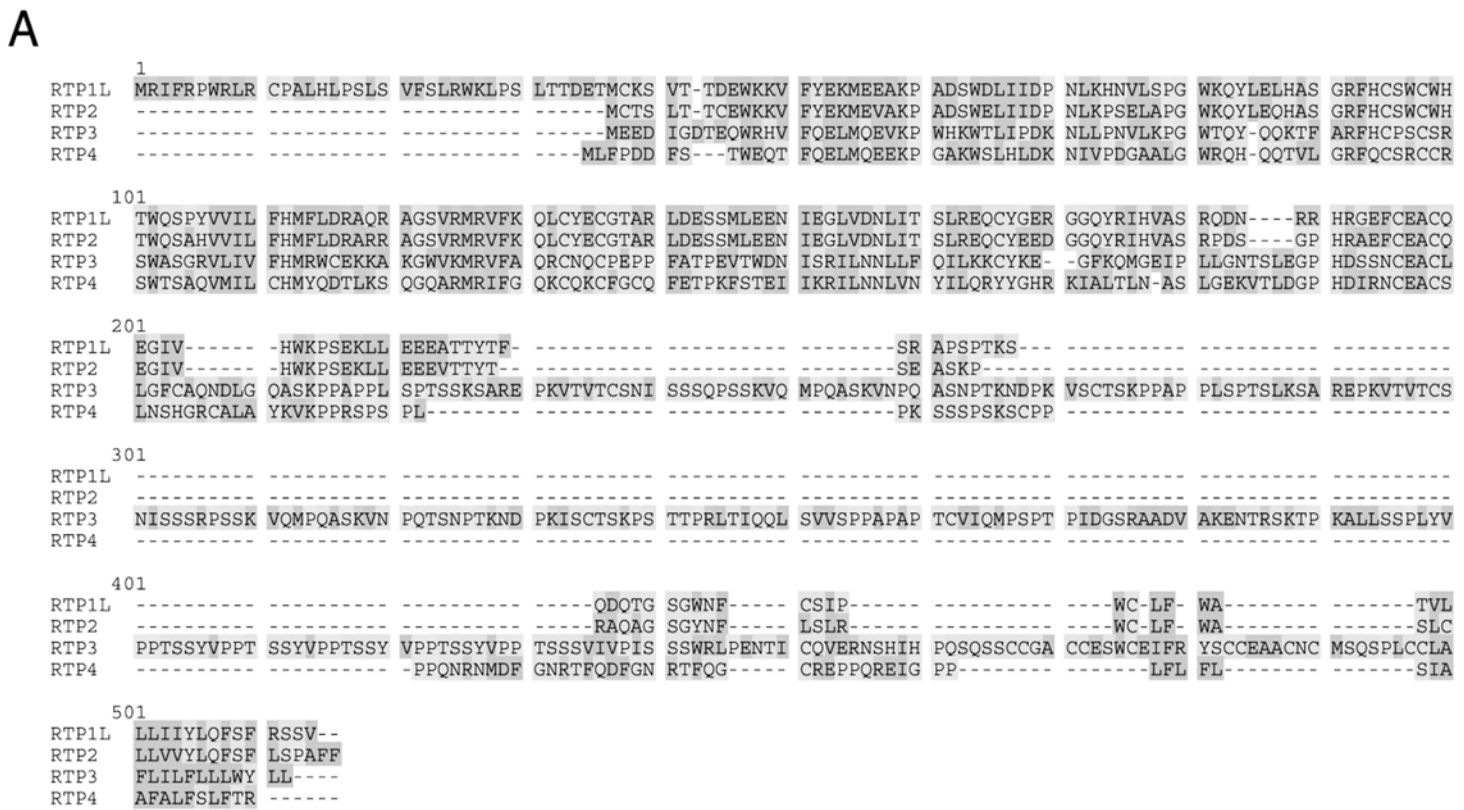
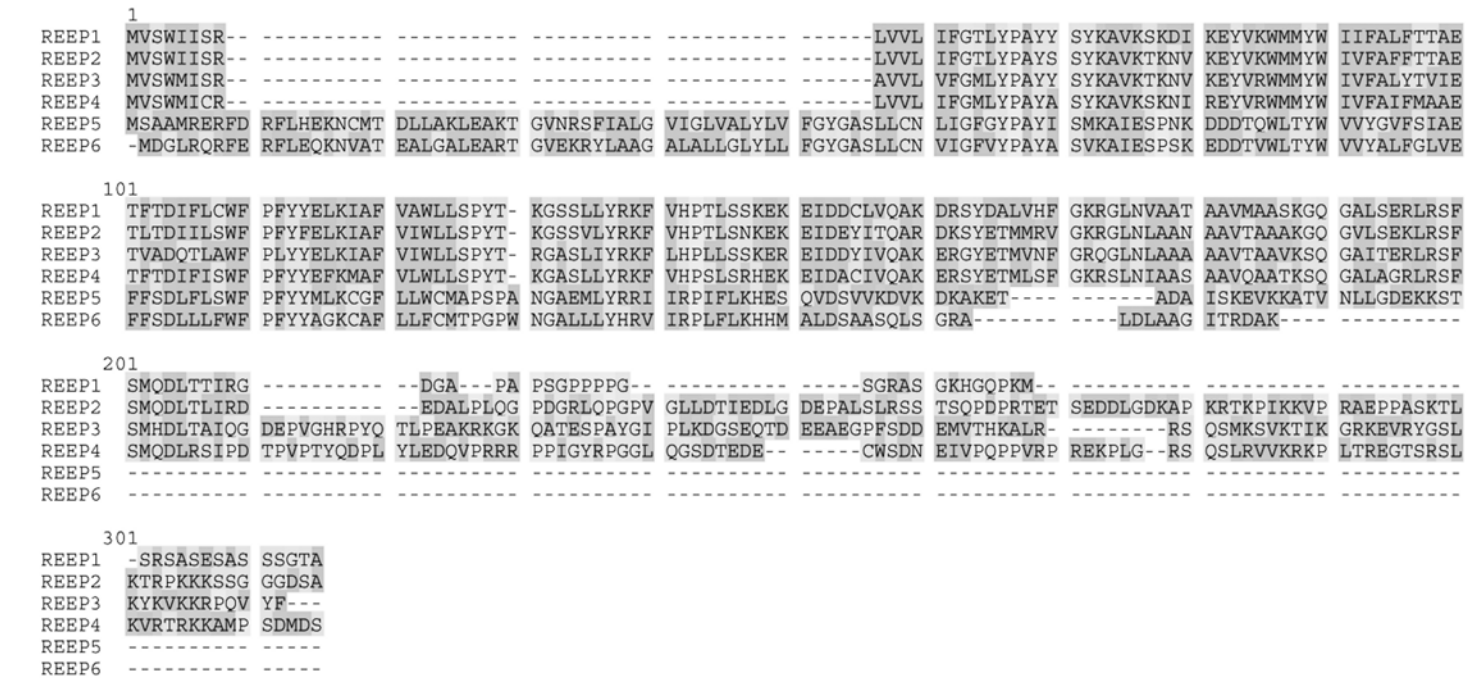



**Figure 1.** Alignment of the A) RTP family and B) REEP family. Physicochemically similar residues are presented in similar colors. A color version of this image is available at www.landesbioscience.com/curie. Figure continued on next page.

B

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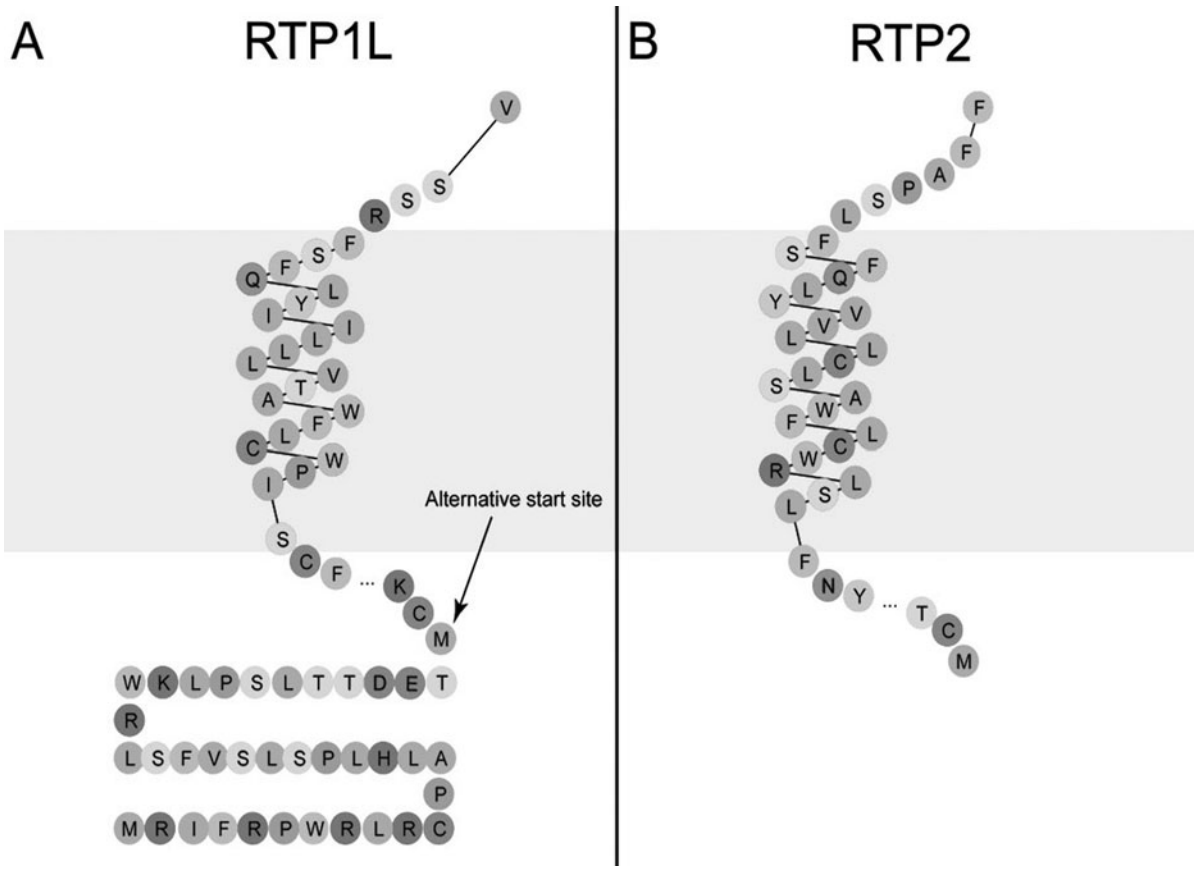


**Figure 2.** Predicted topology of OR accessory factors. A) The second methionine of RTP1L, residue 37, may be the true start site for translation. Receptor expression is higher when the first 36 amino acids are removed from the construct. B) RTP2 aligns with the shortened form of RTP1. C, viewed on following page) REEP1 has two predicted transmembrane domains. Alternatively, the first 19 amino acids may function as a signal peptide. Based on amino acid sequence, SignalP 3.0 indicates that there is a 60% chance the N-terminus of REEP1 functions as a signal peptide. Figure continued on next page.

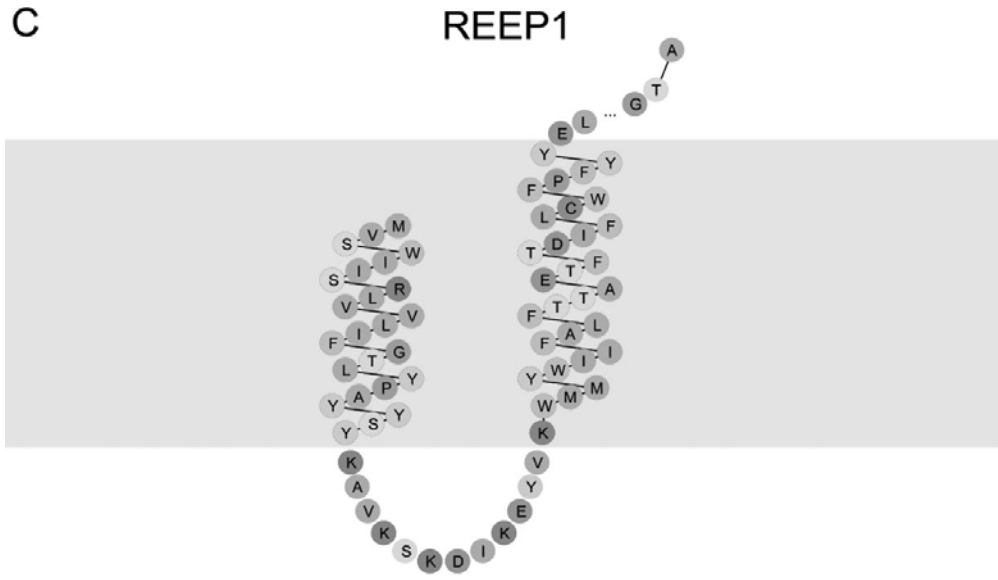


**Figure 2,** continued. See figure legend on previous page.

T1R3 results in the formation of a sweet taste receptor and coexpression of T1R1 and T1R3 results in the formation of an umami (l-amino acid) taste receptor.[16,17] In the case of V2R putative pheromone receptors expressed in the vomeronasal organ, a nonclassical MHC class 1b molecule and β2-microglobulin may form a complex with some V2Rs.[18]

Following this pattern, mammalian ORs are typically retained in the endoplasmic reticulum (ER) in heterologous cells when expressed alone.[19-22] Saito et al[2] used an expression cloning strategy to screen OR partner molecules. Using a SAGE (serial analysis of gene expression) library from single olfactory and vomeronasal neurons, candidate genes encoding membrane associated proteins were isolated and tested to determine if they enhanced surface expression of an OR, MOR203-1, in a HEK293T cell line. From this pool, RTP1 was identified. RTP1 and the related protein, RTP2, are specifically expressed in the olfactory neurons and promote functional cell-surface expression of various ORs in heterologous cells. RTP1 and RTP2 do not reach the cell surface unless expressed with an OR, suggesting a stable complex of ORs and RTPs trafficks to the cell surface. A third molecule REEP1 was found to be expressed at lower levels in the olfactory epithelium and was less effective in increasing functional heterologous expression of ORs.

ORs coexpressed with RTP proteins are functionally active. RTP1, RTP2 and REEP1 did not seem to affect the ligand specificity of two mouse ORs.[2]

One potential concern in the field is if this in vitro analysis accurately reflects the in vivo properties of odorant receptor function. Using calcium imaging techniques, current recording and cell-based assays, many studies have been able to correlate functional responses from intact neurons to that of in vitro OR pharmacology, albeit not perfectly.[23] Oka et al (2006) compared the response profiles of several mouse ORs to cognate ligands using functional imaging of the olfactory bulb against ORs expressed in HEK293 cells with RTP1 proteins. They showed ligand selectivity of the ORs to chemical ligands is comparable, although the responses vary in efficacy. In addition, in vitro activity of human receptors in heterologous cells has been shown to predict human perception for two different ORs,[24,25] also suggesting that the heterologous system at least partly mimics in vivo function.

Although RTPs promote cell surface expression for a wide variety of ORs, they do not enable a high-level of cell surface expression for all ORs. This may reflect a large fraction of nonfunctional genes among the intact ORs.[26,27] Alternatively, there may be additional partner protein(s) required for cell-surface expression of ORs. For one OR, M71 and the closely related OR, M72, complex formation with some non-olfactory GPCRs, including β2 adrenergic receptors and purinergic receptor P2Y1, promotes cell-surface expression in heterologous cells.[28,29] In addition, one heat shock protein, Hsc70t, has been shown to enhance cell-surface expression of at least one OR (OR17-4),[30] presumably by promoting correct folding.

## MECHANISM

Although RTPs and REEP1 have been shown to increase cell surface expression of ORs, TAS2Rs and the opioid receptors, the mechanisms underlying this effect remain elusive. Expression of GPCRs is a complex process involving protein folding, posttranslational modifications and transport through the cellular compartments including the ER and Golgi apparatus. In addition, a large number of sensory GPCRs require homo or heterodimerization for proper targeting to the plasma membrane.

OR proteins are retained in the ER and subsequently degraded in the proteosome when heterologously expressed.[19,21,22] Coexpression of RTP1S with ORs allows the receptor proteins to exit the ER, although the majority of receptor proteins still appear to localize to the Golgi apparatus.[1]

In the case of TAS2Rs, heterologously expressed proteins are localized in the Golgi apparatus. Co-expression of RTP3 or RTP4 appears to promote the exit of TAS2Rs from the trans-Golgi network and enhance the plasma membrane targeting and functional expression of some TAS2Rs. However,

the in vivo role of RTP3 and RTP4 in taste receptor expression requires further investigation because the expression of these molecules are only detected in the basal progenitor cells in the taste buds.[3]

## Promote Correct Folding

Retention of GPCRs in intracellular structures may be caused by incorrect folding. Hsc70t's effect in promoting OR trafficking is presumably achieved by promoting correct folding.[30] Likewise, members of RTPs and REEPs could promote correct folding of GPCRs. In this sense, it is intriguing that the plant homologs of REEP1, HVA22s are induced by stress, resembling the properties of heat shock proteins.[31]

## Facilitate Transport to the Plasma Membrane

A second possibility is that these proteins could facilitate the transport of specific vesicles/cargo that include ORs and/or other GPCRs. Consistent with this idea, a REEP1 homolog in yeast, YOP1P, has been implicated in Rab-mediated vesicle transport.[32] A drosophila homolog of REEP1 (CG30193) interacts with sec23.[33] The yeast sec23 has been implicated in protein transport from the ER to the Golgi apparatus.[34]

## Coreceptor

Finally, these proteins could form a stable complex with the partner GPCRs and act as a coreceptor. This mechanism is implicated by the fact that RTP1 cell surface expression is enhanced by co-expression of ORs.[2] In this scenario, some GPCRs including ORs might contain ER retention signal(s) that are masked by the association with RTPs (or REEPs), a mechanism similar to the regulation of cell-surface expression of GABA(B) R1 receptor by the association of GABA(B)R2.[35-37] Heterodimerization of small number of ORs with other GPCRs can occur and has been shown to increase surface expression.[28,29]

The RTPs and REEPs might have different or even complementary roles, a hypothesis that is consistent with the absence of any amino acid sequence similarity or specific sequence motifs. The three roles outlined above appear to be the most reasonable ones for these proteins; however, other possible functions cannot be excluded. For example, as with RAMPs, the accessory proteins could regulate the glycosylation of the ORs, an important step for OR trafficking.[38,39]

## CROSS-PARTNERING

In addition to the main olfactory system, many vertebrates possess an accessory olfactory system consisting of the vomeronasal organ and a dedicated accessory olfactory bulb. This system appears to be important for detection of some pheromones, chemicals that influence innate behavior or physiology in the same species. Two families of putative pheromone receptors that are expressed in the vomeronasal organ, V1Rs and V2Rs, are notoriously difficult to functionally express in heterologous cells. Like ORs, most V1Rs and V2Rs seem to be retained in the ER when expressed in heterologous cells. Though RTP1 and RTP2 are expressed in the vomeronasal organ, coexpression of the RTP family members with V1Rs or V2Rs does not seem to facilitate trafficking of the receptor proteins in heterologous cells, suggesting that the vomeronasal neurons may use different molecular machinery from that of ORs and other known chemosensory receptors for their trafficking.

## CONCLUSION

The RTP and REEP families appear to promote cell-surface expression of some GPCRs including olfactory and taste receptors and opioid receptors. The mechanism of action is poorly understood at this time. The existence of several closely related family members with disparate phenotypes suggests that identifying important residues through mutational analysis of these proteins may lead to a better understanding of the mechanism of action for these proteins.

It is not known whether RTP and REEP members are required for GPCR trafficking and function in vivo. Generation and analysis of knockout animals will be critically important to assess the in vivo function of these proteins. Additionally, in the case of ORs, these mutant animals may also shed some light on developmental processes in the olfactory system such as axon pathfinding and targeting in the olfactory bulb, as ORs themselves have instructive roles in this process.[40,41]

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CHAPTER 8

# REGULATION OF RAMP EXPRESSION IN DISEASES

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**Abstract:** Receptor-activity modifying proteins (RAMPs) belong to a single family of transmembrane proteins. RAMPs determine ligand specificity of G-protein coupled receptors; calcitonin receptor and the calcitonin-receptor like receptor (CLR). To date, three members of RAMP family (RAMP-1, -2, -3) have been identified. The co-expression of RAMP-1 with CLR constitutes the calcitonin gene related peptide receptor whereas the association of the RAMP-2 or RAMP-3 with CLR forms the adrenomedullin (AM) receptor. Alterations in signaling and subcellular distribution of G-protein coupled receptors can be responsible for the regulation of many disease conditions. These changes may be mediated by the different isoforms of RAMPs associated with such receptors. In this chapter, we describe the differential responses associated with upregulation of RAMPs in disease conditions. For instance, the upregulation of all three RAMP isoforms contributes to the cardioprotective effects of the CLR/RAMP ligands. On the other hand, strong evidence exists for the involvement of AM in various cancers and that its action is mediated by the upregulation of RAMP isoforms, RAMP-2 and -3. Though limited, a few studies have been reported on the differential response associated with the upregulation of RAMP in other disease conditions such as sepsis, liver cirrhosis, glomerulonephritis, Type 1 diabetes and Parkinson's disease. Thus, the regulation of RAMP expression is involved in the pathophysiology associated with various diseases.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

Receptor activity-modifying proteins (RAMPs) belong to a novel family of single-transmembrane-domain proteins. To date, three isoforms of RAMPs, RAMP-1, -2 and -3, have been identified. RAMPs determine the ligand specificity of the calcitonin receptor-like receptor (CLR).[1] The co-expression of RAMP-1 isoform with CLR results in the formation of the calcitonin gene related peptide (CGRP) receptor, whereas the expression of the RAMP-2 forms the adrenomedullin (AM) receptor. The association of RAMP-3 with CLR responds to AM and to a lesser affinity to CGRP.[2-4] The regulation of RAMPs and their ligands are studied extensively in disease conditions such as chronic heart failure, hypertension-induced cardiomyopathy, and various forms of cancer. A few studies have been reported in other disease conditions such as sepsis, kidney diseases, Type 1 diabetes and Parkinson's disease. In this chapter, we describe some of the studies reported in the above disease conditions and highlight the differential responses associated with RAMPs in different pathological realms.

## REGULATION OF RAMP IN HEART FAILURE

Calcitonin gene related peptide and AM are potent vasodilators in humans. The neurotransmitter peptide CGRP is produced by alternative processing of the RNA transcribed from the calcitonin gene. CGRP is present in a large population of cardiac afferents and the atrial myocardium is richly innervated by CGRP nerve fibers.[5-7] Endogenous CGRP may have cardioprotective effects in rats and humans during myocardial ischemia.[8,9] Patients with congestive heart failure showed improved cardiac performance, hemodynamics and renal blood flow after CGRP administration.[10-12] In guinea pigs, CGRP receptors are localized throughout the ventricular myocardium and higher levels of expression were observed in the conducting system, atrial myocardium and intrinsic cardiac ganglia.[13]

AM is a peptide with structural homology to CGRP and is produced in several tissues including the kidneys, lungs and heart.[14,15] Both AM and AM receptors are abundant in the heart and blood vessels.[16] AM acts on myocardial cells and vascular smooth muscle cells to stimulate adenylate cyclase thereby increases intracellular cAMP levels resulting in vasorelaxation.[16,17] Plasma levels of AM have been reported to be increased in hypertension, renal failure, acute myocardial infarction and heart failure in references to the clinical severity of the disorders.[18-20] Administration of AM in humans has been shown to decrease mean arterial pressure in healthy subjects without any adverse effects.[21] Furthermore, short-term infusion of AM increased

cardiac index and decreased mean pulmonary arterial pressure in patients with chronic stable heart failure.[22,23]

In a rat model of heart failure induced by left coronary artery-ligation, AM mRNA expression was increased 1.5 fold in the nonischemic left ventricle with maximal levels at 28 days after induction of myocardial infarction.[24] Likewise, RAMP-2 mRNA expression levels were increased by 1.4-1.6-fold from days 2, 7 and 42 compared to sham operated rats. RAMP-2 mRNA levels in the ischemic region at 16 days after myocardial infarction were 3 times the levels observed in the sham operated rats. This study was the first to demonstrate upregulation of RAMP mRNA during pathological conditions indicating the dynamic control of AM receptor binding activity and selectivity for agonist in diseases.[24] Since there were parallel elevations of the AM mRNA and RAMP-2 mRNA during heart failure, it is suggestive that the receptor expression is not a compensatory response to the increased levels of AM. Therefore, the concerted upregulation of AM, AM receptor and RAMP-2 suggests that the activation of the myocardial AM signaling system plays a significant role in the pathophysiology in post-infarction failure.

In a nonischemic model of chronic heart failure induced by pressure overload due to aortic banding, RAMP-1 and RAMP-3 mRNA and protein expression levels in both atria and ventricles were upregulated at 6 months after aortic stenosis.[25] Surprisingly, no change was detected in CLR, RAMP-2 and AM mRNA expression levels. In that study, there was significant upregulation of RAMP-1 in addition to RAMP-3. Since RAMP-3 isoform has affinity to CGRP, the observed upregulation in RAMP-3 suggest functional roles for both CGRP and AM in compensating chronic heart failure in rats.[25] Significantly increased expression of RAMP-2, CLR and AM mRNA was also found in the atria and the ventricles of rats with congestive heart failure caused by coronary artery ligation.[26]

It has been shown that most patients with heart failure have a history of hypertension and/or left ventricular hypertrophy.[27,28] Hypertension and left ventricular hypertrophy are the most common risk factors for heart failure and they appear to contribute to majority of the heart failure cases.[29] Plasma levels of AM are elevated in hypertension, myocardial infarction and heart failure.[18,19] Cardiac AM and its gene expression has been reported to be upregulated in rats with pressure and volume overload-induced cardiac hypertrophy and myocardial infarction.[30-32] Using a Dahl salt-sensitive rat model in which systemic hypertension causes left ventricular hypertrophy at the age of 11 weeks followed by heart failure at the age of 18 weeks, Nishikimi et al addressed the question whether AM participates in the pathophysiology during the transition from left ventricular hypertrophy to heart failure.[33] Their study showed that AM gene expression was significantly elevated at

the left ventricular hypertrophy stage and was even further increased at the heart failure stage. Moreover, gene expression of AM receptor components, CLR, RAMP-2 and RAMP-3 were significantly elevated at both stages of left ventricular hypertrophy and heart failure. That study strongly suggests that cardiac AM system may modulate the pathophysiology during the transition from left ventricular hypertrophy to heart failure.[33]

AM levels in plasma, myocardium and aorta were augmented in rats with isoproterenol (ISO)-induced myocardial ischemia. AM mRNA in myocardium and aorta was also increased in that model. In addition, RAMP-2 mRNA in myocardium and aorta was increased by 19.6% and 15.8%, respectively, as compared to those of the control group.[34] Yet another study showed that both mRNA and protein amount of AM, CLR, RAMP-1, -2 and -3 in the myocardium of ISO-treated rats were increased.[35] This indicated that changes in cardiac AM/AM receptor may be significant in the pathogenesis of ischemic myocardiopathy.

In several animal models of hypertension including that which induced by the vasoconstrictor peptide angiotensin II (Ang II), CGRP and CGRP receptor expression were increased.[36] Receptor blockade, on the other hand, increased the severity of hypertension in those models.[36,37] These studies showed that endogenous CGRP acts through a negative feedback mechanism to ameliorate the development of hypertension. Ang II-induced hypertension was markedly attenuated in transgenic mice overexpressing the human RAMP-1 gene. Ang II-induced decreases in baroreflex activity and heart rate variability, and increases in blood pressure variability observed in control mice were significantly blocked in transgenic human RAMP-1 mice. This suggests RAMP-1 overexpression induces protective change in cardiovascular autonomic regulation.[38] In contrast, mice deficient in RAMP-1 (RAMP-$1^{-/-}$) were associated with high blood pressure and the activity of CGRP were markedly suppressed in the arteries of these mice. The LPS-induced inflammatory responses of the RAMP-$1^{-/-}$ mice revealed a transient and significant increase in the serum CGRP levels and high serum levels of proinflammatory cytokines, TNF-$\alpha$ and IL-12 compared to the wild type mice. This suggests that CGRP signaling through CLR/RAMP-1 receptors are involved in the regulation of both blood pressure by vascular relaxation and proinflammatory cytokine production from dendritic cells.

In addition to CGRP and AM, a novel CGRP family member, intermedin (also known as adrenomedullin-2), was identified. Like AM, intermedin is expressed in many organs including the gastrointestinal tract, pancreas and lungs.[39-41] Intermedin has been shown to be a multifunctional peptide involved in regulating cardiovascular functions, including potent dilatation of systemic and pulmonary vessels, influencing regional blood flow, and augmenting cardiac contractility. Intermedin has been demonstrated to be a powerful

renal protective agent with pleiotropic effects in an experimental model of hypertensive rats by preventing endothelial loss, kidney damage, inflammation and fibrosis via inhibition of oxidative stress and proinflammatory mediator pathways.[42] As in the case of AM, intermedin has also proven to be a novel angiogenic growth factor involved in neovascularization of the rat ischemic hindlimb induced by femoral artery ligation.[43] Similar to other peptides in the CGRP family, intermedin signals through G-protein coupled receptor complex. It acts as a selective agonist for CLR/RAMP-1 and CLR/RAMP-3 receptors. Intermedin has been shown to protect the myocardium from the deleterious effects in oxidative stress and hypertrophic stimuli.

In a rat model of congestive heart failure, mRNA levels of intermedin and the receptor complex components in the heart were significantly increased. In an ISO-induced myocardial ischemia,[44] mRNA expression of CLR, RAMP-1, -2 and -3 was increased. Exogenous treatment of intermedin$_{1\text{-}53}$, restored myocardial cAMP content, improved cardiac function and attenuated lactate dehydrogenases and malondialdehyde formation indicating the cardioprotective role on intermedin in ISO-induced myocardial ischemia. In the mouse model of myocardial ischemia/reperfusion injury created by ligating the cardiac left anterior descending artery, compared to the control, infarcted mice showed increased levels of mRNA and protein expression of intermedin in plasma and cardiac tissues.[45] The mRNA expression of RAMP-3 was increased very early and the CLR and RAMP-2 mRNA expression were increased later after reperfusion. RAMP-1 mRNA was not changed with ischemia/reperfusion injury.

These above studies collectively demonstrate that upregulation of RAMP-1, -2 and -3 mRNA and protein expression contributes to the cardioprotective effect induced by CLR/RAMP ligands.

## REGULATION OF RAMP IN CARCINOGENESIS

Our current understanding of the biological processes involved in cancer development is that malignant growth is dependent upon a multistep process including basic essential alterations such as evasion from apoptosis, self-sufficiency in growth signals, angiogenesis, and metastasis. AM is a multifunctional regulatory peptide that is overexpressed in cancer cells and aids to develop several of the molecular and physiological features that are considered as the basis of malignant growth. AM was first isolated from an adrenal tumor (pheochromocytoma) in 1993.[15] Since its isolation, it has been shown to be present in many mammalian tissues of normal and malignant settings.[46] AM has shown to be mitogenic in vitro for human cancer cell lines, i.e., lungs, breasts, colon and prostate.[47,48] The expression of AM has been correlated with tumor type and grade, with high expression

in glioblastomas, while low levels were detected in anaplastic astrocytomas and yet barely detectable levels were seen in low grade astrocytomas and oligodendrogliomas.[49] In these tumor cells, AM acts as an autocrine/paracrine growth factor through CLR/RAMP-2 and -3 receptors. Ouafik et al, demonstrated that the density of vessels with lumen was decreased in the anti-AM antibody-treated tumors suggesting AM might be involved in neovascularization and/or vessel stabilization.[49] Ishikawa et al indicated that the AM antagonist [hAM (22-52)] inhibited the growth of pancreatic cancer cells in vivo indirectly by blocking the formation of large functional blood vessels in the tumor tissues.[50] AM also suppresses apoptosis of endothelial cells and it is protumorogenic either by stimulating angiogenesis alone or both angiogenesis and carcinomal cell growth directly.[49-53] Angiogenesis, the sprouting of new capillaries from preexisting blood vessels, is a multistep process that involves migration and proliferation of endothelial cells, remodeling of the extracellular matrix and functional maturation of the newly assembled vessels. In a study using human umbilical vein endothelial cells (HUVEC), treatment with AM promoted migration and invasion in a dose-dependent manner. The action of AM was specific and was mediated by the CLR/RAMP-2 and -3 receptors. AM was also able to induce differentiation of these cells to cord-like structures on Matrigel. That study suggests that the proangiogenic action of AM mediated by the CLR/RAMP-2 and -3 receptors may be associated with enhanced neovascularization and that both AM and its receptors are potential targets for antiangiogenic therapies.[54]

Low oxygen tension is a physiological feature of carcinogenesis. The decrease in oxygen tension is one of the strongest stimuli for the induction of AM. The effect of low oxygen tension or hypoxia on RAMPs has not been conclusive. Differential response for AM and its receptor components during hypoxia were reported. In a human neuroblastoma cell line, IMR-32, expression level of AM was increased by hypoxia while RAMP-2 mRNA was decreased. The decrease in RAMP-2 mRNA and the simultaneous increase in AM due to hypoxia indicated cellular adaptation to hypoxic stress.[55] Another study showed that the three RAMPs and CLR mRNAs expression was insensitive during hypoxia in cultured rat astrocytes.[56] Yet an additional study indicated that lung tissues from rats exposed to hypobaric hypoxia showed upregulation of RAMP-1 and RAMP-3 and no change in CLR and RAMP-2 mRNAs.[57]

A major stimulus for angiogenesis is cellular hypoxia. Hypoxia induces transcriptional activation of genes that alter cellular metabolism and promote neo-angiogenesis. Pancreatic cancers express high levels of vascular endothelial growth factor (VEGF), a potent pro-angiogenic cytokine, indicating a role for tumor proliferation and invasion. Anti-angiogenic strategies targeting VEGF have been shown to reduce pancreatic cancer

growth, spread and angiogenesis. Anti-angiogenic therapies such as targeting of the VEGF and platelet derived growth factor receptors were introduced for highly vascularized tumors, but most cancers eventually progress under these treatments underscoring the need for new therapies. Interestingly, blocking antibodies to VEGF did not significantly inhibit AM- induced capillary tube formation by HUVEC indicating that AM does not function directly through upregulation of VEGF.[54] These findings suggest the proangiogenic action of AM and identified AM and its receptors as potential new targets for anti-angiogenic therapies.

AM has been shown to play an important role in the growth of cultured pancreatic cells. To evaluate the role of AM in human pancreatic cancer, the expression of AM and its signaling components were investigated in pancreatic adenocarcinomas.[58] In that study, using quantitative real time PCR, mRNA levels of AM and CLR were 1.5 and 2.4-fold higher in the adenocarcinomas compared to normal pancreatic tissues. Immunohistochemical analyses revealed expression of AM, CLR, RAMP-1 and RAMP-2, but not RAMP-3 in pancreatic cancer cells. Similar results were observed in pancreatic cell lines. Blocking of CLR decreased invasiveness in 4/5 pancreatic cancer cell lines. These studies suggest that blocking the signaling components of AM have therapeutic potential in pancreatic cancer.[58]

AM is highly expressed in variety of malignant tissues including glioblastoma, pancreatic carcinoma and clear cell renal carcinoma and has shown to play an important role in tumor growth and angiogenesis. A recent study demonstrated that AM, CLR and RAMP-2 were localized in the carcinomatous epithelial compartment of clear cell renal carcinoma while RAMP-3 staining was observed in the inflammatory cells that infiltrated the tumors suggesting a cross-talk between tumor cells and the microenvironment.[59] Under in vitro condition, exogenous AM treatment stimulated cell proliferation, migration and invasion of cultured tumor cells and has shown that the action of AM was mediated by the CLR/RAMP-2 and CLR/RAMP-3 receptors. These findings highlight the importance of AM signaling components in the metastatic processes.[59] Another study indicated that AM is upregulated in human prostate carcinomas and that AM stimulates in vitro growth of prostate carcinoma derived cell lines by enhancing proliferation and decreasing apoptosis. Interestingly, the tumor promoting effect of AM in these tumors is mainly mediated by the CLR/RAMP-3 receptor subtype.[60]

These studies provided strong evidence for the involvement of AM in the development of malignant growth which include the multistep process of evasion from apoptosis, angiogenesis and metastasis and that its action is mediated by CLR/RAMP-2 and RAMP-3 receptors. Whether CGRP

and its receptor, CLR/RAMP-1, have any role in carcinogenesis, however, remains to be elucidated.

## REGULATION OF RAMP IN SEPSIS

Plasma concentrations of AM are markedly increased in acute inflammatory conditions such as sepsis and septic shock.[61-64] The role of RAMP in these inflammatory conditions has not been completely understood. In a murine endotoxemia model of sepsis, Ono et al, observed significant decrease in the expression of CLR and RAMP-2 mRNA in the lungs as compared to normal mice, whereas RAMP-3 gene expression was markedly increased in the lungs, spleen and thymus.[65] The authors suggest that the increase of plasma AM due to endotoxemia is caused by the marked decrease of the CLR/RAMP-2 binding sites for AM in the lungs and that there appears to be a change in receptor subtype, i.e., CLR/RAMP-3 expression for AM in sepsis suggesting distinct role for AM during the clinical course of this syndrome.[65] In contrast, Ornan et al, observed no alteration in the lung CLR or RAMP-2 expression in a rat model of sepsis induced by cecal ligation and puncture (CLP). Interestingly, RAMP-3 expression was significantly upregulated at 5 h after CLP (i.e., early sepsis) but not at 20 h after CLP (i.e., late sepsis). This suggested that the early upregulation of RAMP-3 expression could be a compensatory mechanism to clear the AM from the blood stream.[66] In accordance with these pulmonary data, the gene expression of CLR, RAMP-2 and RAMP-3 were not significantly altered in aortic tissues at 20 h after the onset of sepsis.[67] Therefore, further studies are required to determine the role of RAMPs in sepsis.

The cardiovascular response to sepsis by CLP is characterized as an early hyperdynamic phase which consists of increased cardiac output and tissue perfusion and decreased vascular resistance. This early phase is followed by a late, hypodynamic phase which constitutes decreased cardiac output and tissue perfusion and increased vascular resistance.[68] Studies have shown that AM plays an important role in producing this hyperdynamic phase in sepsis.[69,70] In fact, intravenous infusion of rat AM in normal animals produced the characteristic hyperdynamic response, including increased cardiac output and microvascular blood flow, and decreased total peripheral resistance.[71] However, the reduced vascular responsiveness to AM has resulted in the transition from this early hyperdynamic phase to a late hypodynamic phase in sepsis. This reduction in vascular response in sepsis has shown to be due to the decrease in the AM specific binding protein, AMBP-1 (identical to complement factor, Factor H), rather than a change in the gene expression of the components for the AM receptors.[67,72] Studies have demonstrated that treatment of septic rats with a combination of AM and AMBP-1 prevented

the transition from the early to late phase, maintained cardiovascular stability, decreased pro-inflammatory cytokine release and improved survival.[72,73] In addition to sepsis, treatment with AM/AMBP-1 produced beneficial effect in other acute inflammatory conditions such as hemorrhagic shock, and gut, hepatic and renal ischemia and reperfusion injury.[74-77] Therefore, strong evidence exists for developing AM/AMBP-1 treatment as novel therapy for such inflammatory conditions (Fig. 1).

## REGULATION OF RAMP IN OTHER DISEASES

Though limited, differential regulation of RAMP expression has been implicated in experimental animal models of other diseases, e.g., liver cirrhosis, glomerulonephritis, diabetes and Parkinson's disease. It is well established that plasma AM level is increased in disease conditions that are associated with fluid retention and volume overload which include cardiovascular, respiratory, hepatic and renal disorders.[78,79] A number of studies suggested that arterial vasodilation in liver cirrhosis may be related to the increase in circulating levels of AM and CGRP.[80-83] The clinical feature of liver cirrhosis is associated with hemodynamic changes that are characterized by arterial vasodilation, increased cardiac output and the development of portal hypertension.[80,84] In an animal model of liver cirrhosis induced by carbon tetrachloride, at 6 and 12 weeks after induction, plasma AM levels and gene expression of AM in the liver were markedly increased. At 12 weeks, the gene expression of liver CLR, RAMP-1, -2 and -3 were elevated when compared to control.[85] This suggests that the increase in expression of AM, CGRP and their receptors precedes the development of cirrhosis and that the release of AM in the cirrhotic liver contributes to peripheral vasodilatation in liver cirrhosis.

In addition to the adrenal glands, kidneys also express high levels of AM which have been immunochemically localized in the glomeruli, tubules and collecting duct cells.[86,87] Likewise, CLR and RAMP-2 and -3 are also expressed in the kidney cortex and medulla.[88] Mesangial cells in the kidney produce AM and in vitro, AM levels are increased with response to TNF-$\alpha$ and IL-1$\beta$.[89] Platelet derived growth factor induced mesangial cell proliferation is suppressed by AM.[90] In an experimental model of glomerulonephritis induced by anti-thymocyte serum injection in rats, at day 7 after induction when mesangial cell proliferation peaked, there were no alterations in the gene and protein expression levels of AM in the glomeruli. However, on day 14 when almost all glomeruli had appeared to return to normal, AM and RAMP-2 expression levels significantly increased and by day 28, these levels were decreased to control levels. Since AM and RAMP expression have

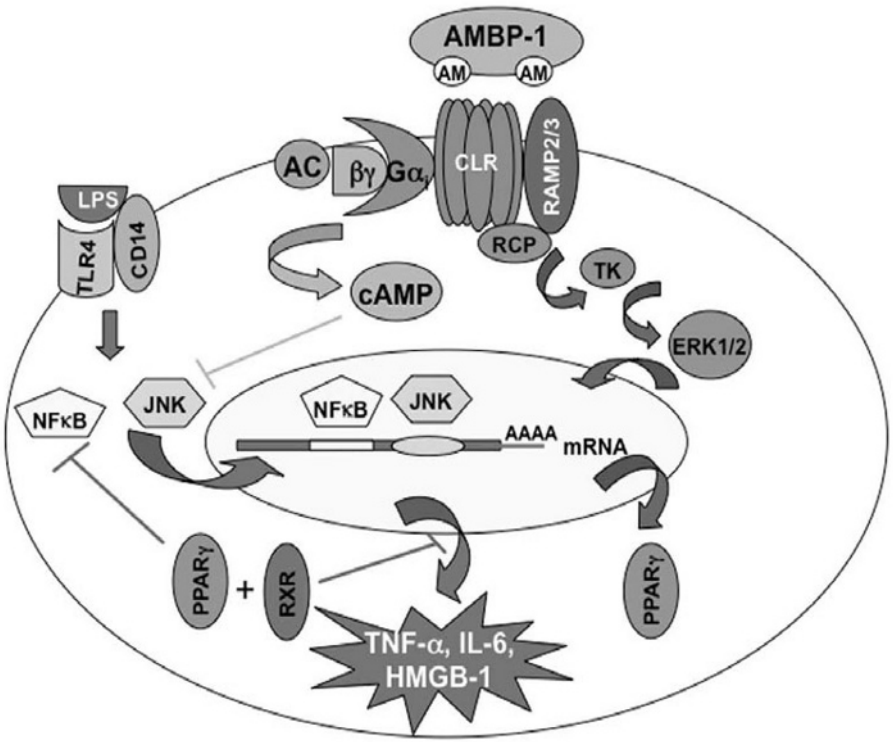


**Figure 1.** The mechanism of RAMP signaling in mediating anti-inflammatory properties in sepsis. AMBP-1 bound AM binds to CLR/RAMP2/3, G-protein coupled receptor(s) and through the activation of adenylate cyclase (AC) leads to the production of cAMP. LPS binds to CD14/TLR4 receptor and upregulates JNK and NF-kB leading to the release of proinflammatory cytokines, i.e., TNF-α, IL-6 and HMGB-1. AM/AMBP-1 induced increase in intracellular cAMP inhibits JNK, resulting in the downregulation of proinflammatory cytokines. The AM/AMBP-1 bound CLR/RAMP2/3 receptor also associates with another protein, RCP, initiates signaling by activation of the Pyk-2 tyrosine kinase (TK) and ERK1/2 which leads to increased PPARγ expression. ERK1/2 induced PPARγ inhibits NF-kB translocation into the nucleus and suppresses proinflammatory cytokine production.

increased at the resolution phase rather than the proliferative phase, AM's action on the mesangial cells translate to amelioration of glomerulonephritis.[91]

In Type 1 diabetes mellitus, persistent hyperglycemia induces progressive and irreversible glomerulosclerosis that ultimately leads to end stage renal failure. Acute intrarenal hemodynamic alterations in response to hyperglycemia are characteristic features of acute phase of diabetic nephropathy in Type 1 diabetes, i.e., increased glomerular capillary volume and selective dilatation of afferent arterioles. The subsequent glomerular hyperfiltration leads to the following progressive renal injury.[92] Using the gene expression profile by microarray approaches, investigators observed that AM gene expression in the kidneys was upregulated in streptozotocin-induced diabetic rats as compared to normal rats.[93] Likewise, RAMP-2 gene expression was also significantly increased in the diabetic kidneys. Upregulated expression of AM and RAMP-2 was observed in afferent arterioles and glomeruli.[94] This upregulation may be related to selective dilatation of glomerular capillary in the acute phase of Type 1 diabetes. In fact, adenoviral delivery of AM to STZ-induced diabetic rats improved cardiac function and prevented renal damages.[95] Therefore, upregulation of AM and CLR/RAMP-2 may be adaptive and protective response in acute hyperglycemic stage as in the acute phase of Type 1 diabetes. However, in the later stage, glomerular hypertension induced by AM and persistent hyperglycemia may lead to endothelial dysfunction and diabetic glomerulosclerosis.[94] Thus, AM and its receptor appear to have distinct functions in the early and late stages of Type 1 diabetes.

Parkinson's disease is a neurodegenerative disorder characterized by the loss of dopaminergic cells in the substantia nigra pars compacta that innervate the striatum.[96] Treatment with L-DOPA provides initial symptomatic benefits in Parkinson's disease but prolonged treatment leads to abnormal involuntary movement known as L-DOPA induced dyskinesia.[97] It is well recognized that RAMPs form complexes with G-protein coupled receptors and play important roles in regulating trafficking, defining the phenotype, and desensitization of various G-protein coupled receptors.[98] Although RAMP expression is regulated in pathological situations in the periphery,[99,100] the role of these proteins in the central nervous system has not been completely understood. RAMP-1 is highly abundant in the dopaminoceptive areas, i.e., striatum, cortex and olfactory tubercles.[101,102] A recent study indicated that RAMP1 gene expression is increased following repeated L-DOPA administration in an experimental animal model of Parkinson's disease, the 6-hydroxydopamine lesioned rat, as compared to controls suggesting that alterations in RAMP-1 activity could lead to the pathophysiology of Parkinson's disease and L-DOPA induced dyskinesia.[103]

## CONCLUSION

It is well recognized that alterations in signaling of G-protein coupled receptors can be responsible for the regulation of many disease conditions. These changes may be caused by the expression of different isoforms of RAMPs associated with these receptors. It is also plausible that the RAMP isoforms are the rate limiting factor for such abnormalities. In this chapter, we provided observations by many investigators on the expression of RAMPs in different disease conditions. Although strong evidence exists for the regulation of RAMPs in diseases such as heart failure and various forms of cancer, the role of these proteins in other disease conditions have only been unraveling over the recent years. It should also be noted that much of these data gathered are from studies in experimental animal models of various diseases. Further preclinical and clinical studies are required to completely understand the function of these proteins in the alteration of G-protein coupled receptor signaling in various diseases and to utilize these proteins as targets in developing therapy for such abnormalities.

## ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (NIH) grant, R01 HL076179 (P.W.).

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CHAPTER 9

# PERSPECTIVES ON SOME RECENT STUDIES ON RAMPs

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**Abstract:** This book on RAMPs covers a number of aspects on the biology of RAMPs. However, due to the uniqueness of some recent studies, they were not covered under a general title. Therefore, in this chapter, we describe three recent studies wherein RAMPs were found to be important players in cancer, hypertension and asthma.

*RAMPs*, edited by William S. Spielman and Narayanan Parameswaran.

## INTRODUCTION

As has been discussed in detail in the previous chapters, receptor activity modifying proteins (RAMPs) play important role in the life cycle of certain GPCRs. By virtue of regulating these GPCR functions, RAMPs have been proposed to modulate many different physiological functions. While some of these proposed roles have been tested using in vivo models, we are still scratching the surface in terms of the role of these proteins that are ubiquitously expressed. While many different aspects of RAMP functions were discussed in previous chapters, here we specifically discuss three studies that were recently published (in the year 2010-11).

## RAMP3 IN CANCER BIOLOGY[1]

In a recent study, by Brekhman et al,[1] the authors describe a role for RAMP3 in cancer metastasis. Using microarray, the authors identified RAMP3 as one of the strongly induced genes in MCF-7 cells in response to over expression of a protein called LOXL2. LOXL2 belongs to the family of enzymes called lysyl oxidase, a group of proteins that catalyze the covalent bond formation between collagen and elastin fibrils, a process that is important in the stabilization of extracellular matrix.[2-4] Lysyl oxidases have been demonstrated to play critical role in tumor cell invasion and tumor progression.[5-10] Importantly, lysyl oxidases have been shown to play a crucial role in the oxidation of lysines on a transcriptional repressor called snail-1, which leads to the E-cadherin downregulation.[11] E-Cadherin is an adhesion receptor that is important for epithelial to mesenchymal transition, a process that is critical for transition of a nonmetastatic tumor to metastatic tumor.[12]

## ROLE OF RAMP3 IN LOXL2-INDUCED EPITHELIAL-MESENCHYMAL TRANSITION

Brekham et al first tested if over-expression or knockdown of LOXL2 would alter RAMP3 expression in MCF-7 cells or MDA-MB-231 and LM2-4 breast cancer cells. Confirming the microarray studies, their results showed that over-expression of LOXL2 increased RAMP3 expression in MCF-7 cells whereas knockdown of LOXL2 down regulated RAMP3 expression. Other studies have shown that inhibition of LOXL2 expression in tumor cells leads to mesenchymal to epithelial transition.[9,11,13] Similar to those studies, Brekham et al also demonstrated that knockdown of LOXL2 in breast cancer

cells leads to morphological changes interpreted to be mesenchymal to epithelial transition. More importantly, when RAMP3 was re-expressed in the knockdown cells, it completely reversed the effects of LOXL2 inhibition. The authors further demonstrate that over expression of RAMP3 does not affect LOXL2 expression, suggesting that RAMP3 functions downstream of LOXL2. Invasiveness of breast cancer cells was also inhibited by LOXL2 knockdown and, re-expression of RAMP3 in LOXL2-knocked down cells led to restoration of invasiveness of the cancer cells.

Consistent with the above studies, knockdown of RAMP3 in breast cancer MDA-MB-231 and LM2-4 cells led to morphological changes that were interpreted to be mesenchymal to epithelial transition. To address part of the signaling mechanism, the authors examined the phosphorylation status of p38 MAPK, which has been shown to be important in the tumor cell invasiveness. Knockdown of either LOXL2 or RAMP3 led to reduced p38 MAPK phosphorylation. Additionally, the authors also showed a decrease in blood vessel density in tumors from cells where RAMP3 expression is inhibited. Taken together, the authors demonstrate that LOXL2 mediates cancer cell invasiveness via RAMP-3 and p38 MAPK (see Fig. 1).

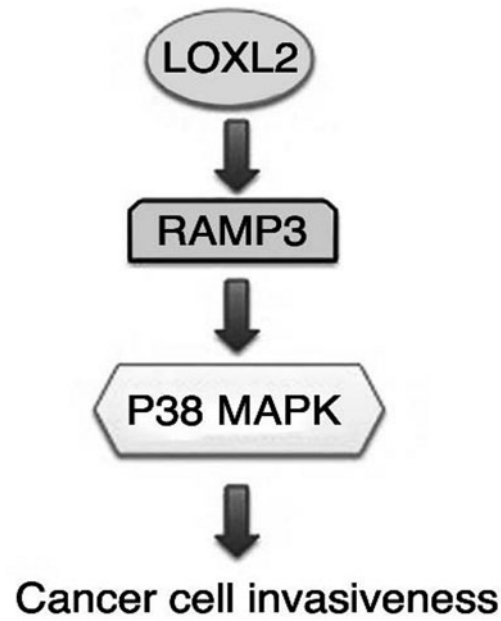


**Figure 1.** Role of RAMP3 in LOXL2-induced cancer invasiveness (see text for details).

## CLR IS NOT INVOLVED IN RAMP3'S EFFECT ON EPITHELIAL-MESENCHYMAL TRANSITION AND TUMOR CELL INVASIVENESS

As discussed in previous chapters, RAMP3 is a partner of CLR, the receptor complex for adrenomedullin. Adrenomedullin is a hypoxia-regulated peptide demonstrated to have angiogenic properties as well as tumorigenic effects. Interestingly, even though MDA-MB-231 and LM2-4 cells produce adrenomedullin, neither of these cell types express CLR, suggesting that the effects of RAMP3 is independent of the classic adrenomedullin receptor. Inspite of an absence of CLR, these cells express calcitonin receptor and unexpectedly, knockdown of CTR, led to inhibition of RAMP3 expression and the consequent pathobiology. Previous studies have shown that CTR in complex with RAMP3 functions as an amylin receptor. However, even though these cells express amylin, antagonizing amylin's actions did not block tumor cell invasiveness or epithelial-mesenchymal transition. Together, these results suggest that RAMP3's effect on tumor cell biology is independent of CLR and may be dependent on CTR and/or other GPCRs.

Although the molecular mechanisms of how RAMP3 mediates LOXL2's effects on epithelial-mesenchymal transition and tumor cell invasiveness are not clearly understood, this study by Brekhman et al is the first to demonstrate a role for RAMP3 selectively in this process. Further studies are obviously necessary to test whether RAMP3 might serve as a direct drug target to prevent epithelial-mesenchymal transition and in tumorigenesis.

## EFFECTS OF RAMP1 OVER-EXPRESSION ON ANGIOTENSIN-INDUCED VASCULAR RESPONSES[14,15]

Calcitonin gene-related peptide (CGRP) is a potent vasodilator with a variety of other functions in the nervous system. Inspite of its vasodilatory effects, studies have shown that systolic or diastolic pressures are not affected by blockade of CGRP receptors in healthy human volunteers.[16] However, in experimental models of hypertension, inhibition of CGRP actions increases the severity of hypertension.[17-20] Based on these and other studies where in CGRP has been shown to play some role in the negative regulation of the development of hypertension, Sabharwal et al,[15] hypothesized that over-expression of RAMP1 in vivo in mice would augment the antihypertensive effects of CGRP. For this, they utilized a mouse model of RAMP1 over-expression, where in human RAMP1 was ubiquitously over-expressed. The authors utilized a transgenic mouse that has been previously generated, in which the loxP sites flank the green fluorescent protein (GFP).[21] In this transgene, human RAMP1 is inserted after the loxP site downstream of GFP. The entire transgene is

under the control of the CX1 promoter, which is a combination of β-actin and cytomegalovirus sequences. They crossed these mice with mice expressing cre-recombinase under the control of adenovirus EIIa promoter (which is expected to be expressed ubiquitously). Consistent with that, the authors found that GFP fluorescence was lost in all the tissues examined, with the exception of some tissues showing patchy fluorescence. The authors also confirmed that the hRAMP1 mRNA is expressed and that it did not affect expression of the endogenous mRAMP1 gene.

Even though under basal condition, the mean arterial pressure (MAP) did not differ between control and hRAMP1 expressing mice, baroreflex sensitivity was significantly increased in hRAMP1 transgenic mice. Furthermore, systolic blood pressure variability was decreased and heart rate variability increased in the hRAMP1 mice. These studies suggest that CGRP indeed plays an important role in cardiovascular autonomic regulation.

Using the same mouse model, the authors then examined the role of RAMP1 in Angiotensin-II-induced blood pressure regulation. For this, they infused Ang-II in control and hRAMP1 mice over a period of several days. They found that while MAP significantly increased in control mice, upon Ang-II infusion, MAP did not increase significantly in the hRAMP1 mice, suggesting that CGRP is able to negatively regulate Ang-II-induced hypertension. In addition, even though diurnal variation in heart rate was almost abolished in control mice after Ang-II-infusion, diurnal heart rate variation was not significantly inhibited in the hRAMP1 transgenic mice. Thus using this unique RAMP1 transgenic mice, the authors have demonstrated that dynamic changes in RAMP1 expression, observed in various diseases including cardiovascular, can potentially affect the pathophysiology of disease. This is especially true for hypertension, where Ang-II system is already an important target. Results from this group suggest that activation of CGRP-RAMP1 system may counteract the Ang-II-induced hypertension and its potential consequences.

Using the same mouse model, Chrissobolis et al[14] tested the hypothesis that over-expression of RAMP1 would enhance the vascular effects of CGRP and that it would also protect against Ang-II-induced vascular dysfunction. Consistent with their hypothesis, CGRP-induced carotid artery relaxation was doubled in hRAMP1 transgenic mice compared to control mice. This effect was indeed specific for CGRP, since relaxation responses induced by adrenomedullin, acetylcholine or U46619 were all similar in control and hRAMP1 mice. Similar to these vascular responses, CGRP-induced vasodilation of basilar artery and cerebral arterioles were significantly enhanced in mice over-expressing hRAMP1 compared to control mice. These enhanced responses were also specific to CGRP similar to that of carotid artery relaxation. Together, these results suggest that RAMP1 plays an important role in CGRP-induced vasodilation and does not play a major role

in adrenomedullin-induced vasodilation in vivo, in these specific vasculatures. Similar to the role of RAMP1 over-expression on Ang-II-induced hypertension, Ang-II-mediated impairment of acetylcholine-induced relaxation of carotid artery was blocked by over-expression of hRAMP1, suggesting that increase in RAMP1 expression levels does negatively regulate Ang-II-induced vascular dysfunction.

In summary, both of the above studies using hRAMP1 over-expressing transgenic mice strongly suggest that the dynamic changes in RAMP1 expression observed in various diseases could potentially have marked pathophysiological consequences.

## RAMP1 IN ASTHMA[22]

Previous studies have shown that CGRP may be involved in the pathogenesis of asthma and cat allergy-induced pathological changes in the airway.[23-26] To further investigate a possible connection between asthma and CGRP, Bonner et al[22] examined the expression levels of CGRP receptor complex in the bronchial tissue between normal and asthmatic human subjects. The authors obtained bronchial biopsies from 15 human patients with mild atopic asthma and 11 normal human volunteers. They performed immunohistochemistry to detect expression levels of RAMP1 protein and in situ hybridization for RAMP1 mRNA in these tissues. Results demonstrated a marked decrease in RAMP1 levels, both at the protein and mRNA levels in bronchial tissues from asthmatic patients compared to normal human volunteers. Using airway epithelial cell lines, the authors also demonstrated that RAMP1 is an important receptor component for CGRP signaling and it is internalized upon continuous stimulation by CGRP. Together, these results demonstrated that RAMP1 is an important component of CGRP receptor complex, and its downregulation may lead to dysregulation in CGRP receptor signaling. The authors propose that the continuous presence of CGRP (as reported in previous studies) may lead to downregulation of RAMP1 in respiratory disease. Even though further studies are necessary to comprehensively examine the consequences of the findings in this manuscript, results from this paper demonstrate that RAMP1 expression is dynamically regulated in asthmatic patients and given the potential role for CGRP in asthma, RAMP1/CGRP receptor complex could be an important drug target in asthma and/or other airway diseases.

## CONCLUSION

"RAMPs" is an emerging field and it is without doubt that we are just scratching the surface with regard to RAMP functions. While many new

functions are to be unveiled in the coming future, studies discussed in this chapter and elsewhere in this book provide a reasonable argument that RAMPs are important drug targets in many different diseases. A further understanding of biochemical and physiological functions of RAMPs in future will undoubtedly lead to new avenues for targeting RAMPs in human diseases.

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