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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.

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]

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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 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.

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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

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