Receptor Activity-modifying Proteins 2 and 3 Have Distinct Physiological Functions from Embryogenesis to Old Age*

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RAMP2/H18528 and RAMP3 target for the specific modulation of GPCR signaling as a pharmacological target. Further exploring the utility of the receptor-RAMP interface as a pharmacological target.

The identification of receptor activity modifying proteins (RAMPs)2 1–3 has revolutionized our current understanding of the mechanism through which class II G protein-coupled receptors (GPCRs) bind to their peptide ligands. First identified in association with the calcitonin receptor-like receptor (CLR; formerly called CRLR), either of these three single pass transmembrane proteins can bind to a GPCR, chaperone it to the plasma membrane, and alter the ligand binding affinity of the receptor (1). For example, a CLR-RAMP1 complex preferentially and specifically binds to calcitonin gene-related peptide (CGRP), whereas a CLR-RAMP2 or CLR-RAMP3 complex will preferentially bind to adrenomedullin (AM), another peptide vasodilator. Thus, the different spatial and temporal expression patterns of RAMP1, RAMP2, and RAMP3 determine how a cell or tissue will sense and respond to either extracellular CGRP or AM.

Biochemical studies using heterologous overexpression of RAMPs in cultured cells have demonstrated that this general mechanism also applies to several other GPCRs of the class II family, including calcitonin receptor, parathyroid receptors 1 and 2, vasointestinal peptide/pituitary adenylate cyclase-activating peptide 1 (VIP/VPAC1) receptor, and glucagon receptor (2, 3). More recently, Bouschet et al. (4) have also demonstrated that RAMP1 or RAMP3 can functionally target a class III receptor, the calcium sensing receptor, to the plasma membrane. Therefore, it is likely that RAMP proteins have evolved to impart a highly controllable mechanism for modulating GPCR signaling that may be broadly applicable to many GPCRs (5). As a consequence, the pharmacological and biochemical study of the RAMP-receptor interaction has been geared toward identifying compounds that exploit this interface as a potential drug target for the specific modulation of GPCR signaling for the treatment of human disease (6). One such compound, BIBN4096BS, which is currently in clinical trials for the treatment of migraine, acts as a selective CGRP antagonist by interfering with the hCLR-RAMP1 interaction (7, 8). Yet, the developmental and physiological consequences of genetically altering RAMP function or expression in the whole animal have not been addressed experimentally.

Receptor-associated RAMPs have been linked with receptor glycosylation, receptor trafficking, ligand binding, and alteration of second messenger signaling (recently reviewed in Refs. 9 and 10). However, these ascribed cellular functions are not consistently conserved among the different receptor-RAMP complexes and are highly affected by the choice of cell type and species of RAMPs studied (9). Moreover, pharmacological studies with the most well characterized receptor-RAMP pairs, CLR-RAMP1–3 and calcitonin-RAMP1–3, demonstrate varying degrees of overlap in the absolute ligand binding affinity imparted by the different RAMPs. For example,
CLR-RAMP1–3 complexes can all bind AM or CGRP but with different affinities (11–13), and calcitonin-RAMP1–3 complexes can form amylin receptors with highly variable affinities (3, 14) or CGRP receptors (15, 16), depending on the cell type studied. Thus, it remains unclear to what degree the three mammalian RAMPs have overlapping functions or whether they can functionally compensate for each other in vivo. To address these questions, we used gene targeting to generate mice with targeted deletions of either the RAMP2 or RAMP3 genes and have determined the effects of their complete absence (in homozygous null mice) and of their reduced expression (in heterozygous mice).

EXPERIMENTAL PROCEDURES

Generation of Mice with Targeted Deletion of the RAMP2 or RAMP3 Gene—To generate the targeting vectors, a 129S6/SvEv genomic library was screened for phage clones containing the 5′ portions of the RAMP2 and RAMP3 genes using DNA fragments isolated from hRAMP2 or hRAMP3 expression plasmids (kindly provided by Dr. Steven Foord, GlaxoSmithKline). Using convenient restriction sites within the genomic clones, 5′ and 3′ regions of homology were subcloned into the multiple cloning site of a gene-targeting vector that contained a phosphoglycerate kinase-neomycin cassette and an herpes simplex virus-thymidine kinase cassette. The final targeting vectors were linearized with NotI before electroporation into embryonic stem cells.

Standard gene targeting methods were utilized to generate embryonic stem cells and mice with a targeted deletion of the RAMP2 gene or a targeted deletion of the RAMP3 gene (17). Briefly, 129S6/SvEv-Tc-1 embryonic stem cells were electroporated with the linearized targeting vectors shown in Figs. 1A and 2A, respectively. After applying positive (G418) and negative (gancyclovir) selection, positive embryonic stem cell clones were identified by Southern blot and/or PCR. The frequency of homologous recombination in the surviving G418/gancyclovir-resistant colonies was 5% for RAMP2 and 1.5% for RAMP3. Male chimeric mice that transmitted the targeted allele were bred to 129S6/SvEv females to establish isogenic lines.

For PCR-based genotyping of the RAMP2-targeted locus, we used the following three primers: primer 1, 5′-CTGAACT-GAACACGGGCA-3′; primer 2, 5′-GGTGACGCCACTG-3′; and primer 3, 5′-GGTCTCTTGGC-3′. Primers 1 and 3 amplify a 1.2-kb-targeted band, whereas primers 1 and 2 amplify a 1.6-kb wild type band.

For PCR-based genotyping of the RAMP3-targeted locus, we used the following four primers: primer 1, 5′-GGCTTACCTCAGGAC-3′; primer 2, 5′-GGTACCTTGAC-3′; primer 3, 5′-GGTCTCTTGGCAAC-3′; and primer 4, 5′-GGGCTAAAGAAGCCA-3′. Primers 1 and 3 amplify a 2.0-kb-targeted band, whereas primers 2 and 4 amplify a 1.4-kb wild type band.

Gene Expression Analysis—RAMP2, RAMP3, and calcrl gene expression was analyzed by quantitative reverse transcription (RT)-PCR with the Mx3000P Q-PCR machine from Stratagene. Primers for RAMP2 amplification were 5′-CAGAATCAATC-TTCATCCACGTGAC-3′ and 5′-GTCATCGAATCTCCTTGT-3′. The probe sequence for RAMP2 detection was 5′-FAM-ATGAAAGACCTACGAAAACATGTCTCTAC-CTTG-TAMRA-3′. Primers for RAMP3 amplification were 5′-GGTCTATTAGAGGCCAGCTGTTG-3′ and 5′-GGGCTAAAC-AAGCCACAGCT-3′. The probe sequence for RAMP3 detection was 5′-FAM-CAGAATCTCAGTGGTCCG-TAMRA-3′. The probe sequence for β-actin detection was 5′-TET-CATGGAACCGAGCGTTCCG-TAMRA-3′. RNA was isolated from adult tissues or embryonic day 13.5 embryos with TRIzol reagent (Invitrogen) and subsequently DNase treated and purified with an RNeasy Mini kit (Qiagen). 200 ng of total RNA was used in each reaction. The ΔΔCt method (19) was used to determine the relative levels of gene expression and shown as a percentage of wild type. All assays were repeated three times, each with duplicates.

Measurement of Basal Blood Pressure, Heart Rate, and Urine Chemistry—Blood pressures and heart rates were measured on unanesthetized mice by a computerized tail cuff system (20). Urine and protein creatinine were measured at the University of North Carolina-Chapel Hill Animal Clinical Chemistry Core Facility.

Experimental Animals—Unless otherwise noted, experimental animals were 4–8 months old and maintained on an isogenic 129S6/SvEv-Tc-1 background. Control animals for all experiments consisted of wild type age- and gender-matched littermates. All experiments were approved by the Institutional Animal Care and Use Committee of The University of North Carolina-Chapel Hill.

Statistics—Statistical analyses for multiple comparisons were performed with one way analysis of variance by JMP Software, SAS Institute. Error bars represent S.E. of the means. Differences were considered significant with a p value of <0.05.
we found no significant compensatory increase in the expres-
sion of either RAMP3 or calcrl in RAMP2−/− mice compared with wild type controls, demonstrating that the
genetic reduction of RAMP2 does not result in a compensatory
up-regulation of RAMP3 or calcrl gene expression.

Generation of Mice Lacking the RAMP3 Gene—Homozygous
null mice for the RAMP3 gene were generated by homologous
recombination using the targeting strategy shown in Fig. 2A. The
disrupted allele, which lacks exons 2 and 3 of the RAMP3 gene
(coding for amino acids 19–147 of 147 total amino acids), was
detected by Southern blot analysis using a genomic probe frag-
ment located outside the areas of homology (Fig. 2B). The correctly targeted allele was further
confirmed by direct sequencing (data not shown). To confirm that
the gene targeting effectively disrupted transcription of full-length
RAMP3 mRNA, quantitative reverse transcription-PCR for
RAMP3 mRNA was performed on total RNA isolated from adult
kidneys. As expected, RAMP3+/− mice expressed approximately
half the wild type RAMP3 RNA levels (45%, p < 0.0001 versus wild
type), whereas RAMP3−/− mice had no detectable levels of
RAMP3 RNA, thus confirming the complete loss of RAMP3 expression in adult homoygous mice (Fig. 2D). To determine
whether genetic deletion of RAMP3 caused a homeostatic com-
penstation in the expression of the RAMP2 or calcrl genes in adult
animals, we measured the expression of these genes in the heart
and kidneys of RAMP3−/− animals. As shown in Fig. 2E, we found
no significant compensatory increase in the expression of either
RAMP2 or calcrl genes in RAMP3−/− mice compared with wild
type controls, demonstrating that the genetic deletion of RAMP3
does not impact on the regulation of RAMP2 or of calcrl gene
expression.
RAMP2 Is Essential for Survival and Normal Fertility—In marked contrast, although the loss of RAMP3 did not affect the survival of RAMP3$^{-/-}$ mice to adulthood, we found that genetic loss of RAMP2 caused embryonic lethality, as no RAMP2$^{-/-}$ pups were born alive to heterozygote matings. This remarkable contrast in phenotypes (embryonic lethality of RAMP2$^{-/-}$ mice versus survival of RAMP3$^{-/-}$ mice) demonstrates that these two genes have significantly different functions during embryonic development.

Offspring from RAMP3$^{+//-}$ matings were born in the expected Mendelian ratio of 1:2:1 (26 RAMP3$^{+/+}$, 48 RAMP3$^{+/-}$: 25 RAMP3$^{-/-}$ pups from 15 litters) and had litter sizes comparable with isogenic control matings (6.6 pups/litter for RAMP3$^{+/-}$ matings versus 6.0 pups/litter for 129S6/SvEv isogenic control matings) (Fig. 3). In contrast, although the ratio of wild type:heterozygote genotypes for viable pups born to RAMP2$^{+/-}$ matings was in the expected Mendelian distribution of 1:2 (22 RAMP2$^{+/-}$:44 RAMP2$^{-/-}$ pups from 29 litters), the average litter size was significantly reduced to 2.1 pups/litter at weaning. This markedly reduced litter size was significantly below the expected litter size of 4.5 pups/litter, which took into consideration the gestational loss of RAMP2$^{-/-}$ null embryos (further confirmed by the assessment of litter sizes for calcrl heterozygote matings in which there was gestational loss of null embryos (Fig. 3) (18). Thus, although genetic loss of RAMP3 was dispensable for normal fertility, a modest genetic reduction of RAMP2 was sufficient to cause marked subfertility, demonstrating that the two genes maintain distinct physiological functions during adulthood.

Aged RAMP3$^{-/-}$ Mice Fail to Gain Weight—RAMP3$^{-/-}$ mice survived to adulthood, reproduced, and displayed no obvious phenotypic defects until ~6 months of age. Although the body weights of young RAMP3$^{-/-}$ mice did not differ from their wild type controls up to ~6 months of age (Table 1), we noticed that older RAMP3$^{-/-}$ mice (9–10 months of age) weighed nearly 9 grams less than age-matched wild type mice (wild type mice weighed 36.1 ± 1.9 g versus 27.3 ± 1.1 g for age-matched RAMP3$^{-/-}$ mice) (Fig. 4B). In contrast, aged RAMP2$^{-/-}$ mice did not differ significantly in body weight from their wild type littermates (wild type weighed 28.3 ± 0.5 g versus 28.8 ± 0.7 g for age-matched RAMP2$^{-/-}$ mice) (Fig. 4A). Despite their visually lean appearance, we found no significant differences in food or water intake in either young or aged RAMP3$^{-/-}$ mice compared with their age-matched wild type controls (Table 1). Moreover, RAMP3$^{-/-}$ mice, similar to their wild type counterparts, survived to at least 18 months of age with no obvious decline in health. Because RAMP3 is highly expressed in the proximal tubule of the kidney, we also compared urine volume and kidney function (as determined by protein:creatinine ratio) between RAMP3$^{-/-}$ mice and wild type controls and found no obvious differences (Table 1). The body weights, feeding behavior, and kidney function of RAMP2$^{+/-}$ mice or calcrl$^{+/+}$ mice did not differ from wild type control littermates (Table 1).

Blood Pressure and Heart Rates Are Unaffected in RAMP2$^{+/-}$ and RAMP3$^{-/-}$ Mice—Because CLR is the best characterized receptor partner for RAMP2 and RAMP3 and because CLR binds to two potent peptide vasodilators (AM and CGRP), we sought to compare the blood pressure and heart rates of RAMP2$^{+/-}$ and RAMP3$^{-/-}$ mice to those of calcrl$^{+/+}$ (18) and wild type mice using a computerized tail cuff system. As shown in Table 1, we found that reduction of RAMP2 to ~50% of wild type levels and the complete absence of RAMP3 had no effect on the basal blood pressure of conscious animals compared with wild type mice or with

**FIGURE 3. Severely reduced fertility in RAMP2$^{+/-}$ mice.** Average litter sizes at weaning resulting from heterozygote matings of the genotype indicated. The number at the bottom of each bar represents the total number of litters. Litter size of calcrl$^{-/-}$ matings is significantly reduced because of the previously reported embryonic lethality of calcrl$^{-/-}$ embryos (18). *p < 0.05 by analysis of variance.

**TABLE 1**

| Phenotypic analysis of RAMP2$^{+/-}$ and RAMP3$^{-/-}$ mice |
|----------------------------------------------------------------|
| Wild type | RAMP2$^{+/-}$ | RAMP3$^{-/-}$ | calcrl$^{+/-}$ |
| Body weight (4–6 mo., g) | 25.8 ± 0.6 | 27.3 ± 0.8 | 24.3 ± 0.5 | 25.6 ± 0.9 |
| Food intake (24 hr, g) | 4.6 ± 0.2 | 4.2 ± 0.2 | 4.1 ± 0.2 | 4.5 ± 0.3 |
| Water intake (24 hr, ml) | 3.6 ± 0.2 | 3.7 ± 0.2 | 3.4 ± 0.1 | 3.4 ± 0.2 |
| Urine volume (24 hr, ml) | 1.0 ± 0.1 | 1.3 ± 0.1 | 1.1 ± 0.2 | 1.0 ± 0.1 |
| Urine protein/creatinine | 0.12 ± 0.02 | 0.12 ± 0.01 | 0.08 ± 0.01 | 0.10 ± 0.03 |
| Mean blood pressure (mm Hg) | 106 ± 5 | 105 ± 3 | 106 ± 5 | 111 ± 5 |
| Mean heart rate (beats/min) | 641 ± 18 | 635 ± 9 | 605 ± 10 | 591 ± 17 |
| Left ventricle/body weight ratio (3–6 months) | 4.11 ± 0.22 | 4.13 ± 0.14 | 3.98 ± 0.14 | 3.97 ± 0.17 |
DISCUSSION

In summary, we used gene targeting to generate two independent mouse lines with deletion of either the RAMP2 or RAMP3 genes. Gene expression analysis in mice with reduced or absent RAMP2 levels or a complete lack of RAMP3 did not reveal any compensatory up-regulation of either RAMP3 or RAMP2 gene expression, respectively, supporting our conclusion that there is no functional redundancy at the transcriptional level between RAMP2 and RAMP3 in vivo. We did observe a general trend for significantly reduced expression of RAMP2, RAMP3, and calcrl compared with wild type mice in the models we tested (Figs. 1D and 2E), which is likely reflective of the high sensitivity of these genes to altered physiological homeostasis (11, 12).

Although the biochemical and pharmacological profiles of RAMP2 and RAMP3 appear to overlap for certain GPCRs (CLR, calcitonin, and VIP/VPAC1), our genetic studies demonstrate that the two genes have distinct roles throughout the life of an animal. During embryonic development, RAMP2−/− mice failed to survive, whereas RAMP3−/− mice appeared normal up to 6 months of age. During adulthood, the loss of RAMP3 had no apparent effect on fertility. In contrast, a modest genetic reduction of RAMP2 in heterozygous mice was sufficient to cause a marked reduction in litter size, which is similar to the phenotype we have previously characterized for mice with a genetic reduction of AM (21). Finally, in aged animals, we found that RAMP3 (but not RAMP2) plays an important role in maintaining normal body weight; however, the physiological mechanisms that account for this phenotype have not yet been resolved.

Our studies to address the regulation of blood pressure and heart rate in these mice are consistent with our recent findings demonstrating that genetic alteration of AM peptide levels from 50–140% wild type levels does not affect basal blood pressure. These results are also consistent with another recent study where transgenic overexpression of mRAMP2 in smooth muscle cells had no effect on basal or induced changes in blood pressure (22). Lu et al. (23) have also shown that genetic deletion of α-CGRP does not alter basal blood pressure in mice. Taken together, our results indicate that in vivo genetic alteration of RAMP2, RAMP3, or calcrl expression (the receptor signaling components required for transducing the signal of two potent vasodilators, AM and CGRP) does not impact on basal blood pressure regulation in mice. Thus, the use of CLR-RAMP2 or CLR-RAMP3 as pharmacological targets for the treatment of hypertension in humans should be carefully evaluated.

Our most significant data stem from the direct comparison of phenotypes for the RAMP2 and RAMP3 gene-targeted mice. Although modest changes in the genetic dosage of the RAMP2 gene have profound effects on survival and reproduction, complete absence of the RAMP3 gene seems to have little or no effect on mice until old age. These in vivo findings are consistent with the concept that RAMP2 acts to mediate the basal effects of normal GPCR signaling, whereas RAMP3 may become induced under physiological conditions (24) or disease (25) to alter the signaling of GPCRs (10). This concept is further supported by biochemical studies that demonstrate that RAMP3 (but not RAMP2) contains an intracellular PDZ motif capable of binding to N-ethylmaleimide-sensitive factor and Na+/H+ exchanger regulatory factor to change receptor internalization and trafficking (26, 27). Thus, the continued study of these genetically engineered mouse models under normal conditions and disease states, as well as cell lines derived from them, will provide useful tools for unraveling the functional role of RAMP2 and RAMP3 in modulating GPCR signaling and testing their potential utility as pharmacological targets for the treatment of human disease.

FIGURE 4. Reduced body weights in aged RAMP3−/− mice (but not aged RAMP2+/− mice). A, body weights of 9–10-month-old RAMP2+/- mice compared with their age-matched isogenic controls (Student’s t test, p = 0.6, n = 7 for each group). B, body weights of 9–10-month-old RAMP3−/− mice compared with their age-matched, isogenic controls (Student’s t test, p < 0.001, n = 10 for each group).

calcr+/− mice. Moreover, we found no overt differences in the heart rates among the genotypes tested (Table 1).
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