RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor

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Calcitonin-gene-related peptide (CGRP) and adrenomedullin are related peptides with distinct pharmacological profiles. Here we show that a receptor with seven transmembrane domains, the calcitonin-receptor-like receptor (CRLR), can function as either a CGRP receptor or an adrenomedullin receptor, depending on which members of a new family of single-transmembrane-domain proteins, which we have called receptor-activity-modifying proteins or RAMPs, are expressed. RAMPs are required to transport CRLR to the plasma membrane. RAMP1 presents the receptor at the cell surface as a mature glycoprotein and a CGRP receptor. RAMP2-transported receptors are core-glycosylated and are adrenomedullin receptors.

The calcitonin family of peptides comprises five known members: calcitonin, amylin, two calcitonin-gene-related peptides (CGRP1 and CGRP2) and adrenomedullin (ADM) (Fig. 1). Calcitonin is involved in the control of bone metabolism and is also active in the central nervous system (CNS). Amylin also has specific binding sites in the CNS and is thought to regulate gastric emptying and have a role in carbohydrate metabolism. The CGRP peptides differ from each other by three amino acids and have so far proved to be indistinguishable in their biological activities. These activities include the regulation of neuromuscular junctions, of antigen presentation within the immune system, of vascular tone and of sensory neurotransmission[1,2]. ADM, the most recently discovered member, is also a potent vasodilator[1,2]. ADM has specific receptors on astrocytes[3] and its messenger RNA is upregulated in CNS tissues that are subject to ischaemia[4].

The calcitonin family peptides probably act through seven-transmembrane-domain, G-protein-coupled receptors (GPCRs). The gene encoding the calcitonin receptor has been cloned[5]. The calcitonin receptor is homologous to GPCRs in 'family B', which typically recognize regulatory peptides such as secretin, glucagon and vasoactive intestinal polypeptide (VIP). Later, CRLR was identified[6,7]. It has 55% overall identity with the calcitonin receptor. Other members of the calcitonin peptide family were candidate ligands for CRLR but CRLR seemed not to be a CGRP receptor[8]. Two related members of the 'family A' class of GPCRs, RDC1 and G10D, were then identified as receptors for CGRP and ADM, respectively[9,10]. This was unexpected, as although RDC1 and G10D are homologous to each other, they are very different from the calcitonin receptor; conversely, CGRP, ADM and calcitonin are related. Later, a cytosolic protein, RCF (receptor component factor), from the guinea-pig organ of Corti was expression-cloned because of its ability to induce CGRP-mediated responses in *Xenopus laevis* oocytes[11]. Finally, a single HEK293 cell line engineered to express CRLR also bound to CGRP and showed CGRP-mediated responses[12].

Isolation and cloning of RAMP1

We chose to clone the gene encoding the human CGRP receptor by an expression-cloning strategy using a SK-N-MC cell complementary DNA library. SK-N-MC cells are derived from a human neuroblastoma, bind ^{125}I-labelled CGRP1 and respond to CGRP by increasing levels of intracellular cyclic AMP[13]. The cDNA library was transcribed *in vitro* and pools of complementary RNA were injected into *Xenopus* oocytes with cRNA encoding the cystic fibrosis transmembrane regulator (CFTR). CFTR contains a cAMP-activated chloride channel that can be used as a sensitive read-out for receptors that couple positively to adenylyl cyclase[14]. Human CGRP1 was used throughout and is referred to as CGRP.

Xenopus oocytes have an endogenous CGRP receptor[15], detected through the CFTR as a small inward current after CGRP application (23 ± 15 nA at -60 mV, $n = 15$). Similar, small CGRP responses were recorded in most oocytes expressing the pools of SK-N-MC cRNA. However, from a single pool CGRP responses were recorded that were larger than the endogenous response. This pool of clones was subdivided repeatedly according to their CGRP responses and a single cDNA was isolated that encoded a 148-amino-acid protein which we have called receptor-activity modifying protein 1 (RAMP1). In oocytes expressing RAMP1, large, dose-dependent responses to CGRP (1330 ± 315 nA, $n = 15$) were recorded (Fig. 2, inset). The responses were inhibited by the CGRP receptor antagonist $CGRP_{8-37}$, with an estimated pA_2 of 8.4, typical of a type 1 CGRP receptor[1] (Fig. 2). RAMP1-expressing oocytes showed no significant responses to ADM, calcitonin or amylin. *Xenopus* oocytes contain endogenous adenosine, VIP and β-adrenergic receptors, each of which will couple to the CFTR, but expression of RAMP1 had no effect on the responses of these receptors when they were activated (data not shown). In this system, RAMP1 appears to be specific for CGRP-mediated responses.

The hydrophobicity plot of the RAMP1 protein indicates that it has an amino-terminal signal sequence and single putative transmembrane domain close to the carboxy terminus (see below). This

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ADM       YRQSMNNFQGLRSFGCRFGTCTVQKLAHQIYQFTDKDKDNVAPRSKISPQGY
CGRP1               ACDTATCVTHRLAGLLSRSGGVVKNNFVPTNVGSKAF
CGRP2               ACNTATCVTHRLAGLLSRSGGMVKSNFVPTNVGSKAF
AMYLIN              KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY
CALCITONIN          CGNLSTCMLGTYTQD-----FNKFHTFPQTAIGVGAP
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Figure 1 The amino-acid sequences of the calcitonin family peptides, aligned for comparison.

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was unexpected as we thought that the CGRP receptor would be a seven-transmembrane-domain GPCR. However, RAMP1 is not, by itself, a CGRP receptor. The effector concentration for half-maximal response (EC_{50}) of 10 nM for CGRP in RAMP1-expressing oocytes is at least tenfold lower than that for the CGRP receptor in SK-N-MC cells. Expression of RAMP1 in mammalian cells (human embryonic kidney (HEK)293T, COS7 or Swiss3T3 cells) did not induce cAMP responses to CGRP or specific binding to ^{125}I-labelled CGRP1. We speculated that RAMP1 induced CGRP-mediated responses in oocytes because it could potentiate the endogenous CGRP receptor. RAMP1 might thus be expected to potentiate a mammalian CGRP receptor in the same way.

CRLR requires RAMP1 for functional expression

The GPCRs RDC1 and CRLR have been reported to be receptors for CGRP. We expressed both GPCRs in *Xenopus* oocytes, but neither altered the endogenous response to CGRP (Fig. 3a). In the same experiments, oocytes expressing the calcitonin receptor responded well to calcitonin (>500 nA; data not shown). When CRLR was expressed with RAMP1, a larger CGRP response was recorded than if RAMP1 had been expressed alone ($3,111 \pm 806$ nA versus $1,330 \pm 315$ nA; Fig. 3). In contrast, expression of RDC1 with RAMP1 did not alter the oocytes' responses to CGRP (data not shown).

We studied the interaction between CRLR and RAMP1 in HEK293T cells (a subclone of HEK293 cells that has been engineered to express the SV40 large T antigen). HEK293T cells do not express endogenous CGRP or calcitonin receptors (unlike some HEK293 lines)[16]. Neither RAMP1 nor CRLR induced significant responses to CGRP when transfected alone, but expression of both produced cells that responded to CGRP by increasing intracellular cAMP levels and binding specifically to ^{125}I-labelled CGRP1 (Fig. 3b, c). RAMP1 and CRLR reconstituted a CGRP receptor that displayed virtually identical pharmacology to the CGRP receptor in SK-N-MC cells (Table 1) and in a CRLR-expressing cell line[12]. RDC1 (ref. 10) did not induce binding or responses to CGRP in HEK293T cells, with or without co-expression of RAMP1 (data not shown).

The requirement for CRLR and RAMP1 to reconstitute a CGRP receptor explains why it has been difficult to expression-clone, its failure to function in oocytes, and the observation that CRLR can only function as a CGRP receptor in certain cell lines[8,12,16] (which presumably express endogenous RAMP1; see below).

Mechanism of RAMP1 activity at CRLR

We proposed that CRLR represents the CGRP receptor and that RAMP1 transports it to the cell surface. To test this, we analysed HEK293T cells transiently transfected with Myc-epitope-tagged CRLR by fluorescence-activated cell sorting (FACS). Four per cent of the cells transfected with Myc-CRLR showed significant cell-

Table 1 Characterization of CGRP and ADM receptors in recombinant and native cell lines

	^{125}I-CGRP1 binding* IC_{50} (nM)		^{125}I-rat ADM binding† IC_{50} (nM)	
	SK-N-MC	CRLR–RAMP1	NG108-15	CRLR–RAMP2
Peptide				
CGRP1	0.1	0.1	1,966	1,300
CGRP2	0.01	0.01	130	300
ADM_{1-52}	0.4	9	0.21	0.75
ADM_{13-52}	3	100	25.7	17
ADM_{22-52}	600	400	60	15
$CGRP_{8-37}$	0.8	0.3	120	75
Amylin	NSE	NSE	NSE	NSE
Calcitonin	NSE	NSE	NSE	NSE

* Binding of ^{125}I-labelled CGRP1 was determined in membranes prepared from SK-N-MC cells and from Swiss 3T3 cells constructed to express CRLR and RAMP1. Typically, the specific binding of ^{125}I-labelled CGRP1 was 3,500 and 1,500 c.p.m. for SK-N-MC and Swiss 3T3 membranes, respectively, using 50 μg of membrane protein per point.
† Binding of ^{125}I-labelled rat ADM was determined in membranes prepared from NG108-15 cells and from HEK293T cells transiently transfected with cDNA encoding CRLR and RAMP2. 20 μg membrane protein was used from NG108-15 cells and 10 μg protein from HEK293T cells. Typically, the specific binding of ^{125}I-labelled rat ADM was 2,000 and 4,000 c.p.m. for NG108-15 and HEK393T membranes, respectively. Binding data were analysed by nonlinear regression using the computer program Allfit. NSE, no significant effect. All determinations were made three times and s.e.m. are less than 10% of the mean in each case.

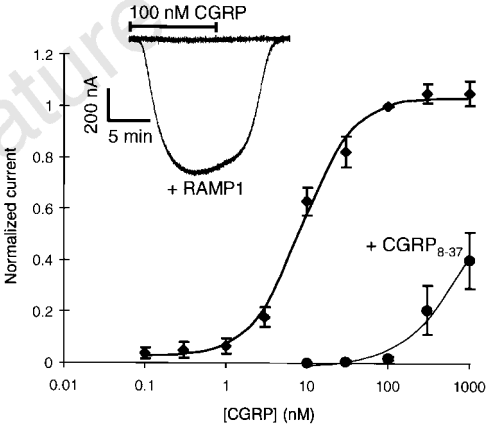

Figure 2 *Xenopus* oocytes expressing RAMP1 show large and dose-dependent responses to CGRP. Oocytes were injected with CFTR cRNA alone or in combination with RAMP1 cRNA. Recordings were made 4 days post-injection. Inset shows responses produced by 100 nM CGRP (bar) at a holding potential of –60 mV (inward current shown by downward deflection). Main graph shows dose–response curves for peak inward current under control conditions and in the presence of 100 nM $CGRP_{8-37}$ (all in the presence of RAMP1 and CFTR). The data from each oocyte were normalized according to their responses to 100 nM CGRP. From curves fitted to these data, we calculated EC_{50} values as 9 ± 1 nM for CGRP (control) and $1,400 \pm 400$ nM (with 100 nM $CGRP_{8-37}$). RAMP1 was selective for CGRP over human amylin, calcitonin and ADM, $n = 4$–7. All data are mean ± s.e.m.

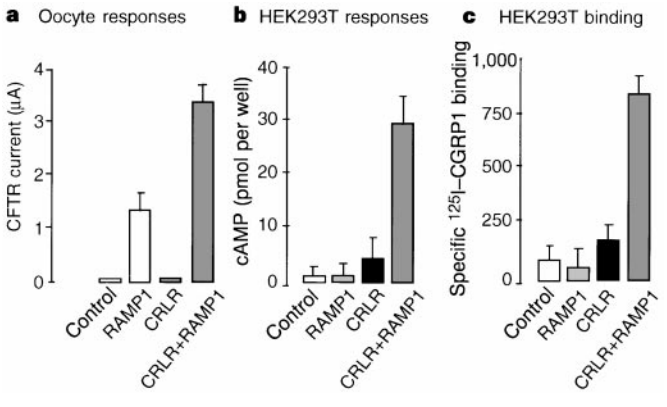

Figure 3 Expression of RAMP1 with CRLR in oocytes and HEK293T cells. **a**, In oocytes, CGRP elicits a small response through an endogenous receptor (control) that is markedly potentiated on expression of RAMP1. CRLR has no effect when expressed alone but allows very large responses when expressed with RAMP1. **b**, In mammalian HEK293T cells there is no endogenous CGRP receptor. RAMP1 alone has no significant ability to produce a CAMP response to CGRP, and neither does CRLR when transfected alone. Expression of RAMP1 with CRLR in HEK293T cells conferred cAMP responses to CGRP. **c**, Specific binding of ^{125}I-labelled CGRP1 to plasma membranes from HEK293T cells transfected with the same cDNAs and at the same time as those shown in **b**.

surface immunoreactivity. This increased to 22% if RAMP1 was expressed with Myc-CRLR (Fig. 4a, top). In the reverse experiment, HEK293T cells were transiently transfected with Myc-epitope-tagged RAMP1, Myc-RAMP1 could not be detected on the cells unless they were permeabilized. However, when CRLR was expressed with Myc-RAMP1, more than 40% of the cells expressed Myc-RAMP1 on the surface (Fig. 4b, bottom). Following permeabilization, 70% of cells showed immunoreactivity towards Myc-RAMP1 or Myc-CRLR, indicating high transfection efficiency. Thus, expression of RAMP1 and CRLR leads to both proteins being presented at the cell surface.

We performed crosslinking studies with the chemical bis[sulphosuccinimidyl]suberate (BS^3) with the aim of irreversibly linking ^{125}I-labelled CGRP1 to its receptor on the surface of HEK293T cells transfected with RAMP1 and CRLR. We studied whole cells before solubilization and analysis on SDS–polyacrylamide gel electrophoresis (SDS–PAGE). As expected, ^{125}I-labelled-CGRP1 could be crosslinked only to the surface of cells transfected with both proteins. After SDS–PAGE, ^{125}I-labelled CGRP1 was crosslinked to two proteins with relative molecular masses (M_r) of 66K and 17K (Fig. 4c). Both associations could be prevented by incubation with 1,000 nM unlabelled CGRP. An M_r of 66K is consistent with that of the native human CGRP receptor[17,18]. The smaller, 17K band[17] could be RAMP1. The crosslinking of ^{125}I-labelled CGRP1 to both proteins indicates a close association, as BS^3 can crosslink primary amine groups that are ~20 Å away from each other.

HEK293T cells expressing Myc-CRLR produced a distinct band of 58K after SDS–PAGE and immunoblotting (Fig. 5a, c) and no 66K band. Myc-RAMP1 ran as a monomer of M_r 14K. At higher expression levels, multimers of Myc-RAMP1 could be detected even under reducing conditions. Expressing Myc-CRLR with increasing amounts of Myc-RAMP1 caused the 58K band to disappear and a diffuse band of about 66K to appear (Fig. 5a). The 66K band size was consistent with that of the protein crosslinked to ^{125}I-labelled CGRP1 on the surface of cells transfected with RAMP1 and CRLR (Fig. 4c). The appearance of the 66K band correlated with specific binding of ^{125}I-labelled CGRP1 to cell membranes (Fig. 5b). We thought that RAMP1 might be required for the terminal glycosylation of CRLR.

Endoglycosidases F and H can be used to differentiate between *N*-linked glycoproteins. When the 58K band (representing CRLR expressed in the absence of RAMP1) was treated with endoglycosidase F, a single immunoreactive species of M_r 48K was seen after SDS–PAGE. Thus, the 58K form is a glycoprotein (Fig. 5c, lane 2). The 66K form (derived from expression of CRLR with RAMP1) was also reduced to a 48K form by treatment with endoglycosidase F, showing that the additional mass units gained when RAMP1 is expressed with CRLR represent carbohydrate residues (Fig. 5c, lane 3). The 66K form is resistant to endoglycosidase H, indicating that CRLR has been terminally glycosylated, an event normally associated with transit through the Golgi complex and the production of mature glycoproteins (Fig. 5c, lane 6). The 58K form is sensitive to endoglycosidase H, indicating that it has not been terminally glycosylated. The mechanism of RAMP1 activity is consistent

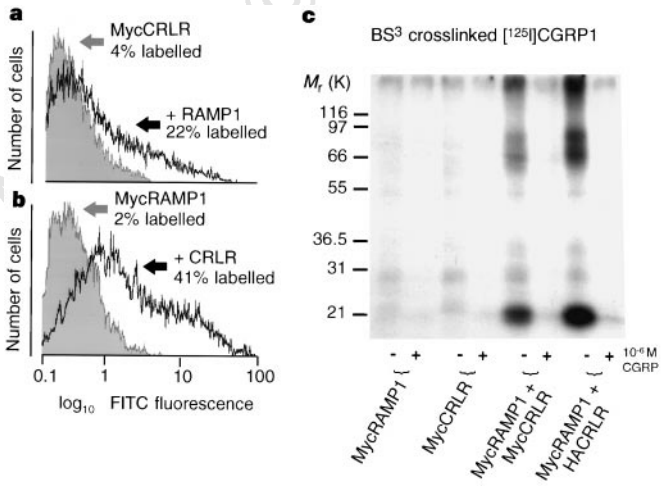

Figure 4 RAMP1 and CRLR are localized together on the cell surface when they are co-expressed. **a**, FACS analysis of HEK293T cells transiently expressing Myc-epitope-tagged CRLR (1 μg ml^{-1} cDNA) with or without RAMP1 (5 μg ml^{-1} cDNA). The number of cells sorted as showing cell-surface Myc-CRLR increased from 4% to 22% of total when RAMP1 was co-transfected. **b**, FACS analysis of Myc-epitope-tagged RAMP1 transfected cells with or without CRLR. The number of cells sorted as showing cell-surface Myc-RAMP1 increased from 2% to 41% of total when CRLR was co-transfected. **c**, Transfection of CRLR with RAMP1 enabled BS^3 specifically to crosslink ^{125}I-labelled CGRP1 to proteins of 66K and 17K on the surface of intact HEK293T cells. CRLR and RAMP1 alone had no effect. Similar results were obtained with HA-tagged CRLR.

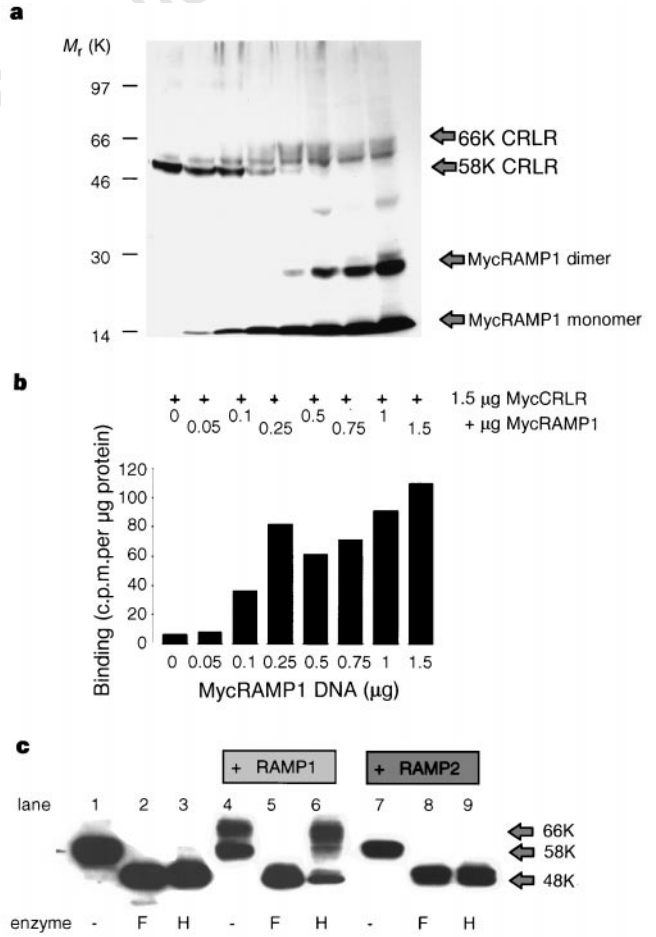

Figure 5 RAMP1 acts to increase the relative molecular mass of CRLR at the same time as specific binding to ^{125}I-labelled CGRP1 becomes apparent. Cells were collected 48 h after transfection with 1.5 μg Myc-CRLR or with Myc-CRLR and increasing amounts of Myc-RAMP1. P2 particulate membrane fractions were prepared for reducing SDS–PAGE, followed by immunoblotting with anti-Myc antiserum (**a**) or by radioligand binding studies with ^{125}I-labelled CGRP1 (**b**). **c**, The 58K CRLR species (produced without RAMP1) is a glycoprotein that is reduced by either endoglycosidase F or endoglycosidase H to a 48K band. The 66K species produced after co-expression with RAMP1 reduced to a 48K band after endoglycosidase F treatment but was resistant to endoglycosidase H, indicating that it is a mature glycoprotein.

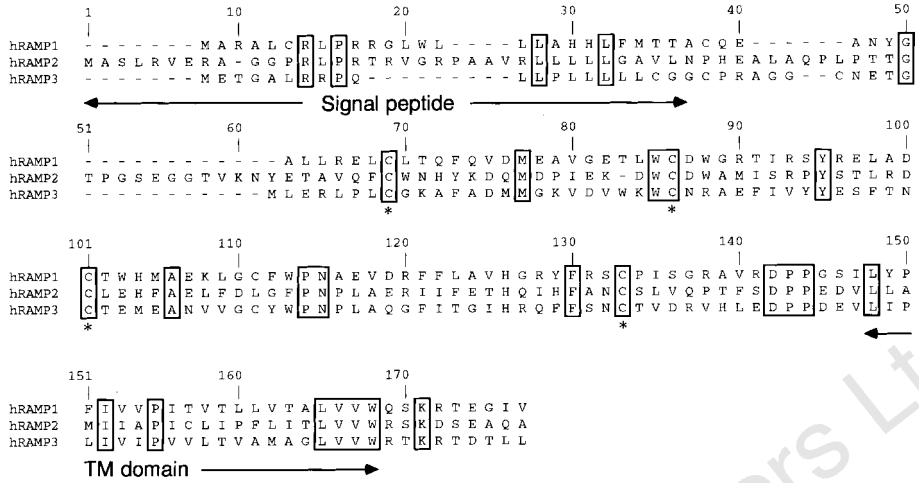

Figure 6 Amino-acid sequences of human RAMPs 1, 2 and 3, aligned for comparison. Putative signal sequences and transmembrane (TM) domains are indicated. Conserved amino acids are boxed and conserved cysteine residues are indicated by asterisks.

with a transport event: CRLR functions as a CGRP receptor after it has been presented at the plasma membrane as a mature glycoprotein. However, during this process RAMP1 is also expressed at the plasma membrane and it may contribute more directly to the function of the CGRP receptor.

The RAMP family of proteins

The human RAMP1 sequence was partially represented by a number of expressed sequence tags (ESTs) in public databases. Searches of these databases also led to the discovery of the partial sequences of two further RAMP1-like proteins, which we have named RAMP2 and RAMP3. A full-length RAMP2 cDNA was isolated from the SK-N-MC cell cDNA library by colony hybridization using a probe amplified by polymerase chain reaction (PCR). A full-length RAMP3 cDNA was isolated by conventional and rapid amplification of cDNA ends (RACE) PCR from human spleen mRNA. Figure 6 shows the amino-acid sequences of human RAMP1, RAMP2 and RAMP3, aligned for comparison. The RAMPs are about 31% identical and about 56% similar to each other. We have identified rodent homologues of RAMPs, but have been unable to find invertebrate homologues.

The hydrophobicity plots of the RAMP amino-acid sequences are very similar, despite the low sequence homologies. In the N termini, four cysteine residues are conserved, suggesting some common secondary structure. RAMPs are type I transmembrane proteins, with an extracellular N terminus and a cytoplasmic C terminus, an orientation confirmed by FACS experiments (Fig. 4b). Both RAMP1 and RAMP3 have a pair of basic residues close to their short, cytoplasmically orientated C terminus. This pair of basic residues is similar to an endoplasmic-reticulum-retrieval motif[19]. However, RAMP2 has a basic–acidic pair of residues rather than a dibasic pair at this position. The functional importance of these residues will be tested.

RAMP mRNA cell and tissue expression

We prepared northern blots using mRNA extracted from SK-N-MC, Kelly (another human cell line derived from a neuroblastoma) and HEK293T cells. Of these cell lines, only SK-N-MC cells exhibit specific binding to ^{125}I-labelled CGRP1 or cAMP responses to CGRP[13] (see Fig. 3 for HEK293T cells; data not shown for Kelly cells). A CRLR-specific probe revealed the expected transcripts[12] of 7.5, 5.5 and 3.5 kilobases (kb) in SK-N-MC cells, but no transcripts in Kelly or HEK293T cells (data not shown). The blots were also hybridized with probes specific for RAMPs 1, 2 and 3. A single transcript of 0.8 kb was seen for RAMP1 and RAMP2 in each cell line; however, RAMP3 mRNA was detected in Kelly cells only (Fig. 7a). Quantification of the RAMP1 and RAMP2 transcripts showed that SK-N-MC and Kelly cells express higher levels of RAMPs 1 and 2 than do HEK293T cells, and that the ratio of RAMP1 to RAMP2 was greatest in SK-N-MC cells.

Figure 7b shows multiple human tissue northern blots examined with the same probes. The transcript sizes for RAMP mRNAs are the same as seen in the cell lines. The RAMP1 gene is expressed in many tissues, including the uterus, bladder, brain, pancreas and gastrointestinal tract. RAMPs 2 and 3 have similar, but not identical, tissue distributions and are expressed strongly in the lung, breast, immune system and fetal tissues.

The data from the cell lines show that RAMPs are not necessarily expressed with CRLR. The distributions of RAMP1 and CRLR

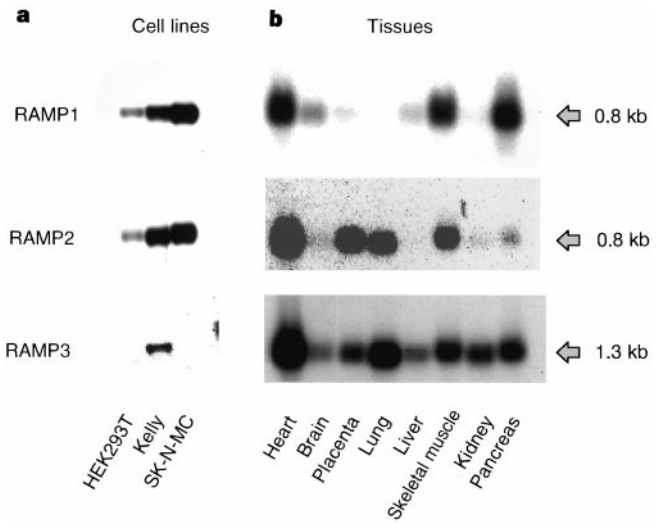

Figure 7 A northern blot analysis of mRNA encoding RAMPs 1, 2 and 3 in cell lines and tissues. **a**, Northern blots of mRNA extracted from human HEK293T, Kelly and SK-N-MC cell lines and hybridized to probes specific for RAMPs 1, 2 and 3. Of these cell lines, only SK-N-MC cells showed either responses to CGRP or CRLR transcripts. mRNA loading was equivalent for each cell line, as judged by comparable expression of glyceraldehyde 3-phosphate dehydrogenase (not shown). **b**, Multiple tissue northern blots representing human tissues, probed as described above.

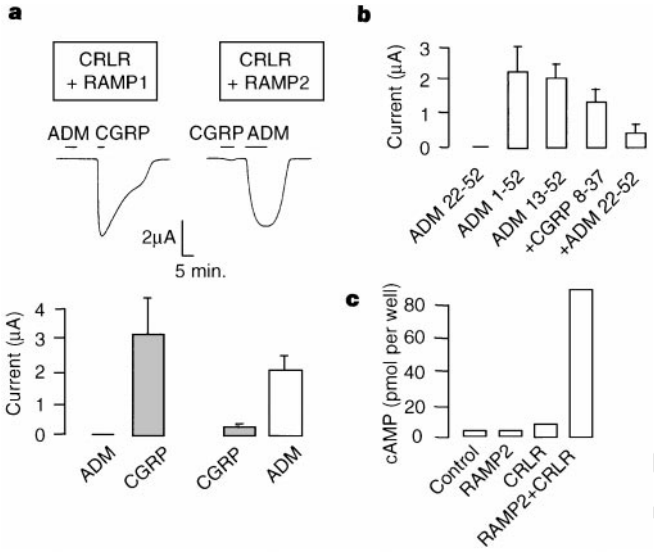

Figure 8 Expression of RAMP2 plus CRLR in oocytes and in HEK293T cells. **a**, Top, representative traces showing responses to 10 nM ADM_{13-52} and 100 nM CGRP in oocytes expressing RAMP1 (left panel) and RAMP2 (right panel) plus CRLR. Bottom, cumulative data from at least eight oocytes for each peptide. Expression of CRLR with RAMP1 in oocytes produced robust responses to 100 nM CGRP but no significant response to 10 nM ADM_{13-52}. In contrast, oocytes from the same batches expressing CRLR with RAMP2 showed robust responses to 10 nM ADM_{13-52} and small responses to 100 nM CGRP. **b**, A pharmacological analysis of the ADM receptor produced in oocytes by expression of CRLR with RAMP2. ADM_{1-52} and ADM_{13-52} are effective agonists whereas ADM_{22-52} and $CGRP_{8-37}$ are not agonists and significantly antagonized the response to 10 nM ADM_{13-52} at 100 nM. *n* was 4–8 oocytes for each treatment. **c**, RAMP2 and CRLR expressed together in HEK293T cells allow new cAMP responses to 10 nM ADM_{13-52}. RAMP2 and CRLR had no effect when expressed alone.

overlap in many tissues and *in situ* localization of the mRNA encoding the two proteins should define which cells express CGRP receptors. CGRP receptors are found in many tissues and always at relatively low levels. High levels of mRNA encoding CRLR[6,12,16] are found in the lung; however, the levels of CGRP receptors in the lung are unremarkable. We detected little RAMP1 mRNA in the lung; in contrast, RAMP2 and RAMP3 mRNAs were particularly abundant. We therefore examined interactions between CRLR and the other RAMP proteins.

RAMP2 and CRLR generate an ADM receptor

In contrast to RAMP1, RAMPs 2 and 3 were unable to potentiate the oocyte response to CGRP. Similar results were obtained in HEK293T cells, in which neither RAMP2 nor RAMP3 allowed the CRLR to function as a CGRP receptor. However, we demonstrated by FACS analysis that RAMPs 2 and 3 were as effective at transporting epitope-tagged CRLR to the cell surface as was RAMP1 (data not shown). SDS–PAGE analysis of epitope-tagged CRLR expressed with either RAMP2 (Fig. 5c) or RAMP3 (not shown) showed mainly 58K proteins and not the 66K form associated with binding to ^{125}I-labelled CGRP1 and responses to CGRP. This 58K species was reduced to a 48K species by treatment with endoglycosidase F or H. Thus, RAMP2 facilitates the transport of CRLR to the plasma membrane, but CRLR transported is a 58K, endoglycosidase-H-sensitive glycoprotein.

As RAMPs 2 and 3 enabled a form of CRLR that is not a conventional CGRP receptor to be presented at the cell surface, we proposed that the receptor might be activated by another ligand. We studied ADM for several reasons: first, ADM and CGRP are related peptides (Fig. 1); second, there is evidence for the existence of both CGRP and ADM receptors in the lung (where CRLR, RAMP2 and RAMP3 are co-expressed); and finally, receptors have been described that are sensitive to both ADM and CGRP[1,2,20–23]. Oocytes expressing RAMP2 and CRLR (plus CFTR) responded to ADM with large inward currents at concentrations of ADM_{13-52} (residues 13–52 of ADM) as low as 0.3 nM, but CGRP produced only small responses (Fig. 8a). The responses to ADM_{1-52} and ADM_{13-52} were similar. ADM_{22-52} and $CGRP_{8-37}$ had no agonist activity (at 100 nM) but significantly antagonized the response to 10 nM ADM_{13-52} (Fig. 8b). No responses were observed to either calcitonin or amylin at 300 nM (not shown).

Expression of RAMP2 failed to generate responses to ADM without CRLR (not shown). This may be because RAMP2 cannot interact with the *Xenopus* homologue of CRLR or because the *Xenopus* receptor expressed with RAMP2 does not recognize

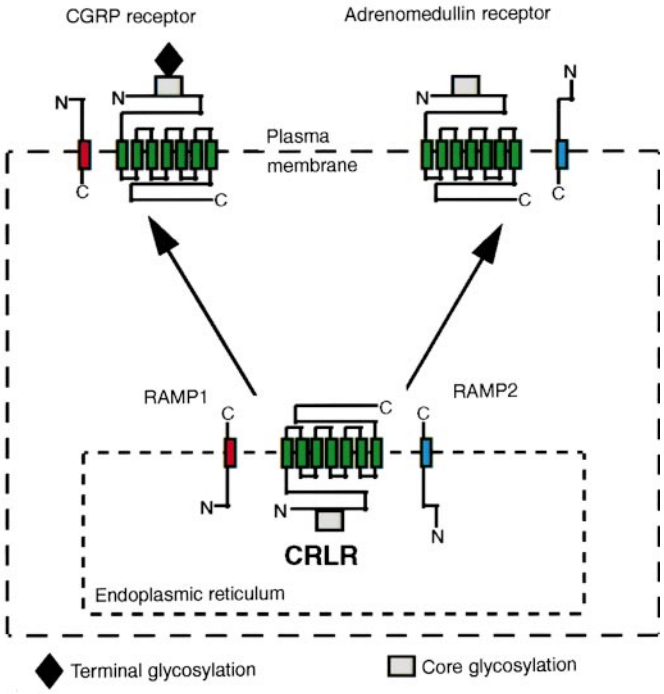

Figure 9 The role of RAMPs 1 and 2 and CRLR in generating CGRP or ADM receptors.

human ADM. In either case, the new response appeared to result entirely from exogenous RAMP2 and CRLR.

Expression of CRLR with RAMP2 in HEK293T cells generated new ADM-stimulated responses of cAMP (Fig. 8c) and specific binding to ^{125}I-labelled rat ADM (Table 1). The absolute levels of specific binding of ^{125}I-labelled rat ADM to co-transfected HEK293T cells and to the native ADM receptors in NG108-15 cells were similar. We constructed competition curves for binding of ^{125}I-labelled rat ADM to membrane preparations from both cell lines in the same set of experiments (Table 1). The half-maximal inhibitory concentrations (IC_{50} values) are similar in each case and are consistent with the pharmacology of the reconstituted ADM receptor in oocytes. Some interspecies variation might be expected as NG108-15 cells derive from a rat–mouse cell fusion[3].

The receptor generated by expression of RAMP3 and CRLR is similar to the ADM receptor and is being studied.

Discussion

We have shown that the CRLR has two alternative pharmacological profiles that are conferred by the accessory proteins RAMP1 (producing the CGRP receptor) and RAMP2 (producing the ADM receptor). RAMPs control the transport and glycosylation of the CRLR (Fig. 9). The differences in pharmacology between RAMP1–CRLR and RAMP2–CRLR could be due to the differential glycosylation of CRLR or to the presence of the RAMP at the plasma membrane (or possibly both). The RAMPs and CRLR can be closely associated and are expressed together at the cell surface. RAMPs 1 and 2 are endogenous to at least three cell lines. Expression of CRLR (either from the native gene or by a recombinant route) with RAMPs would generate a pharmacology dependent on the proportion of CRLR that is transported by each RAMP. A 'pure' CGRP receptor or ADM receptor population might only exist in cells that express CRLR with a single RAMP. However, in practice there is some consensus regarding 'typical' CGRP receptor and ADM receptor pharmacological profiles, and these are represented by the receptors native to SK-N-MC and NG108-15 cells, respectively. The pharmacologies of CGRP and ADM receptors in tissues are less clear, but the responses can be complex as many cells and cell types contribute receptors.

Attempts to identify calcitonin and amylin receptors gave some results similar to ours. These two receptors have similar protein backbones[24] and attempts to clone the gene encoding the amylin receptor using a selective ligand resulted in the isolation of a cDNA encoding the calcitonin receptor[25]. Production of amylin receptors from this cDNA was cell-line-specific (however, calcitonin receptors were always synthesized, with a variable proportion of receptors having amylin receptor pharmacology)[25]. Our data might allow a molecular understanding of some of the complexities associated with the pharmacology of calcitonin and amylin as well as of CGRP and ADM.

The existence of RAMPs indicates a new mechanism whereby cells/tissues could change their responsiveness to different neuropeptides. Whether this is a regulatory event remains to be determined. There will be considerable interest in whether RAMPs regulate other receptor systems. Many receptors are not easily expressed in commonly used host cell lines: $GABA_b$, eotaxin (CKR3)[26], thrombin (PAR3) and olfactory receptors are just a few examples of such receptors. RAMPs may have a wider role, as they are expressed at relatively high levels in many tissues and can be expressed independently of CRLR.

We have now unambiguously identified the CGRP and ADM receptors, described the RAMP family of proteins, and presented a new mechanism for regulating GPCRs that may have physiological importance. □

Methods

Oocytes and expression cloning. We injected capped cRNA into stage V–VI defolliculated oocytes[14]. Each oocyte received 50–100 ng CFTR RNA and 1–20 ng cloned RNAs or 100–400 ng SK-N-MC pool RNA and was tested for responses to CGRP after 3–14 days, under two-electrode voltage clamp. We used the endogenous oocyte β-adrenergic receptor to control for CFTR expression and to ensure that any increased responses were specific to CGRP. A pool of about 0.7 million clones produced a CFTR-mediated response to CGRP. Six further subdivisions were made, corresponding to pool sizes of ~50,000, ~20,000, 4,000 (gridded), 168, 40 and 2 clones respectively. Oocytes were superfused with a Ca^{2+}-free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5 at 25 °C) at a flow rate of 2 ml min^{-1}. Recording electrodes had resistance of 0.5–1.0 MΩ when filled with 3 M KCl. Human ADM peptides (residues 1–52, 13–52 and 22–52) were obtained from Peninsula Labs. Amylin, calcitonin, CGRP2, $CGRP_{8-37}$ and VIP were obtained from Bachem and CGRP1 was synthesized at GlaxoWellcome.

Molecular biology. CRLR[8] was modified to provide a consensus Kozak sequence as described[12]. Myc and haemagglutinin (HA) epitope tags were used in-frame to the 5′ end of cDNAs encoding RAMP1 and CRLR. In each case, we removed the native signal sequence and replaced it with that for CD33 (ref. 27) (Myc-CRLR) or T8 (HA-CRLR). Myc-RAMP1, Myc-CRLR and HA-CRLR were cloned into pcDNA3 (Invitrogen). All modified sequences were compared to 'native' sequences in the assays described and found to behave identically (data not shown). Human RDC1 was cloned by PCR from genomic DNA and canine RDC1 was provided by A. Clarke[10].

Cell culture and cDNA transfection. HEK293T and Swiss3T3 cells were maintained in DMEM medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine and maintained at 37 °C and 95% humidity. NG108-15 cells were cultured in DMEM/HAM F12 (1:1) supplemented with hypoxanthine–aminopterin–thymidine (HAT) (1×) and 10% FCS.

Stable Swiss 3T3 cells expressing CRLR and RAMP1 were generated by dual hygromycin and neomycin selection imposed on cells transfected with plasmids encoding both proteins and different selection markers. Individual lines were cloned by dilution.

5 μg linearized DNA per plasmid was transfected for each T 75 cm^2 flask containing 80% confluent cells. Cells were left for 24 h before plating into 96-well plates and were then cultured for a further day. For cAMP determinations, the cells were washed with PBS and preincubated in DMEM medium containing 300 μM IBMX (Sigma) for 30 min at 37 °C. CGRP or ADM was added for a further 30 min and the cells were washed with ice-cold PBS. cAMP levels were determined using scintillation proximity based assays (Amersham).

Radioligand binding, crosslinking analysis, SDS–PAGE and western blotting. HEK293T cells were collected after 2 days (NG108-15 cells were collected when confluent) into PBS. Cells were pelleted by centrifugation and homogenized in 50 mM HEPES-KOH, pH 7.6 (containing 15 μg ml^{-1} aprotinin, 0.25 μg ml^{-1} antipain, 0.25 μg ml^{-1} leupeptin, 0.1 μg ml^{-1} bezamidine and 0.1 μg ml^{-1} bacitracin). After centrifugation at 500 *g* for 20 min at 4 °C, the supernatant was removed and centrifuged at 48,000*g* for 30 min at 4 °C. The final pellet was resuspended in homogenization buffer and the protein content measured. SK-N-MC and CRLR/RAMP1 double stable Swiss 3T3 cell lines were collected into PBS but subjected to the same membrane preparation in 50 mM HEPES-KOH, 1 mM EDTA, 100 μM leupeptin, 25 μg ml^{-1} bacitracin, pH 7.6. Immediately before the first homogenization, 1 mM phenylmethylsulphonyl fluoride (PMSF) and 2 μM pepstatin A were added. The final pellet was resuspended without PMSF or pepstatin A.

For the CGRP receptor assay, membranes (50 μg) were incubated for 90 min at 25 °C in binding buffer (50 mM HEPES-KOH, 10 mM $MgCl_2$. 1 mM EDTA, pH 7.4), containing 30 pM ^{125}I-labelled CGRP1 (Amersham) in a total volume of 200 μl. For the ADM receptor assay, membranes (10–20 μg) were incubated for 30 min at 4 °C in the same buffer with 100 pM ^{125}I-labelled rat ADM (Amersham) in 200 μl. Incubation for both assays was terminated by rapid filtration through GF/C filters soaked in 0.1% polyethylenimine using a Tomtek cell harvester. Nonspecific binding was determined using a final concentration of either 1 μM CGRP or 1 μM ADM_{13-52}.

For crosslinking analysis, cells were transfected with cDNA and, after two days, detached and incubated with ^{125}I-labelled CGRP1 with or without unlabelled CGRP and crosslinked at 4 °C with either BS^3 or DSS (disuccinimidyl suberate)[17] (not shown but similar results) (reagents from Pierce). The cells were centrifuged and then extracted into sample buffer[17] and subjected to

SDS–PAGE and autoradiography. For other experiments, plasma-membrane-containing P2 particulate fractions were prepared from transfected cell pastes that had been stored at −80 °C following collection. Membrane protein (75 μg) was subjected to 10% SDS–PAGE. Epitope tags were visualized by immunoblotting with anti-Myc or anti-HA monoclonal antibodies and developed using enhanced chemiluminescence (Amersham).

FACS analysis. HEK293T cells were transiently transfected with cDNA as described, collected 2 days later and washed three times in PBS. The cells were resuspended in DMEM and incubated with the primary antibody, 9E10 (anti-c-Myc), diluted 1:30 for 15 min. Following three further washes, the secondary antibody (sheep anti-mouse Fab_2 coupled with fluorescein isothiocyanate) diluted 1:30 was incubated for 30 min in the dark. For permeabilized cells, the Fix and Perm kit (Caltag) was used. FACS analysis was performed on an EPICS Elite (Coulter, Florida). 10,000 cells were sorted in each experiment.

Deglycosylation was carried out according to the supplier's protocols (Boehringer) before SDS–PAGE/immunoblotting.

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Correspondence and requests for materials should be addressed to S.M.F. (e-mail: smf3746@ggr.co.uk). The cDNA sequences for human RAMPs 1, 2 and 3 have been submitted to the EMBL database under the accession numbers AJ001014, AJ001015 and AJ001016, respectively.