CGRP-RCP, a Novel Protein Required for Signal Transduction at Calcitonin Gene-related Peptide and Adrenomedullin Receptors*

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It is becoming clear that receptors that initiate signal transduction by interacting with G-proteins do not function as monomers, but often require accessory proteins for function. Some of these accessory proteins are chaperones, required for correct transport of the receptor to the cell surface, but the function of many accessory proteins remains unknown. We determined the role of an accessory protein for the receptor for calcitonin gene-related peptide (CGRP), a potent vasodilator neuropeptide. We have previously shown that this accessory protein, the CGRP-receptor component protein (RCP), is expressed in CGRP responsive tissues and that RCP protein expression correlates with the biological efficacy of CGRP in vivo. However, the function of RCP has remained elusive. In this study stable cell lines were made that express antisense RCP RNA, and CGRP- and adrenomedullin-mediated signal transduction were greatly reduced. However, the loss of RCP did not effect CGRP binding or receptor density, indicating that RCP did not behave as a chaperone but was instead coupling the CGRP receptor to downstream effectors. A candidate CGRP receptor named calcitonin receptor-like receptor (CRLR) has been identified, and in this study RCP co-immunoprecipitated with CRLR indicating that these two proteins interact directly. Since CGRP and adrenomedullin can both signal through CRLR, which has been previously shown to require a chaperone protein for function, we now propose that a functional CGRP or adrenomedullin receptor consists of at least three proteins: the receptor (CRLR), the chaperone protein (RAMP), and RCP that couples the receptor to the cellular signal transduction pathway.

G protein-coupled receptors are generally thought to function as monomers that interact with G proteins to initiate signal transduction. However, it has recently been recognized that many G protein-coupled receptors require additional proteins for function. These proteins range from other receptors that form dimers, to heterologous accessory proteins that function primarily as chaperones (1, 2). In this study we report a novel accessory protein that does not act as a chaperone, but instead couples the receptor to the cellular signal transduction pathway. Thus, our concept of a G protein-coupled receptor involves a complex of proteins that are required for receptor function, including correct intracellular sorting, organization in the plasma membrane, and coupling to cellular signal transduction proteins.

Calcitonin gene-related peptide (CGRP) is a potent vasoactive neuropeptide, which has been implicated in vasodilation, migraine, and chronic pain (3–6). Despite the clinical implications of CGRP’s biological actions, therapeutic strategies targeting CGRP have been hindered by the lack of a functional CGRP receptor. CGRP binding results in increased intracellular cAMP levels (7, 8), and a candidate G protein-coupled receptor has been identified called the calcitonin receptor-like receptor (CRLR) (9). However, CRLR was initially non-functional when transfected into mammalian tissue culture cells (10, 11). An accessory protein named the CGRP-receptor component protein (RCP) was cloned in our laboratory and found to confer CGRP receptor function in Xenopus laevis oocytes (12). However, co-transfection of CRLR and RCP into tissue culture cells did not yield functional CGRP receptors. A second accessory protein was subsequently cloned, the receptor activity modifying protein (RAMP1), which did yield functional CGRP receptors when co-transfected with CRLR into cell culture (13). In these experiments RAMP1 functioned as a chaperone for CRLR, and was required for expression of CRLR on the cell surface. The function and requirement for RCP has been less clear. RCP has no homology to RAMP1 or other sequences in GenBank, and contains no obvious protein motifs that predict its function. RCP is expressed in CGRP-responsive tissues, and RCP expression correlates with the potency of CGRP in vivo (14–16), but the lack of a requirement for RCP in cell culture co-transfection studies has remained puzzling.

In these studies we determined the role of RCP in CGRP-mediated signal transduction. We made stable cell lines which express antisense RCP and show that RCP is an intracellular peripheral membrane protein that interacts with the CGRP receptor CRLR and facilitates CGRP and adrenomedullin-mediated signaling. RCP thus represents a new class of proteins that facilitate signal transduction at G protein-coupled receptors.

EXPERIMENTAL PROCEDURES

Cloning of RCP Antisense cDNA—To maximize the hybridization between the antisense message and the endogenous RCP mRNA, the

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1 The abbreviations used are: CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RCP, receptor component protein; PAGE, polyacrylamide gel electrophoresis.
NIH3T3 RCP cDNA was isolated. Primers designed against mouse RCP (14) were used for 5’ and 3’ rapid amplification of cDNA ends (Marathon RACE, CLONTECH, Palo Alto, CA), and the full-length RCP cDNA was constructed using methods previously described (17). The NIH3T3 RCP was then cloned in the antisense orientation in the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) and the full-length RCP cDNA was inserted using the MMessage mMachine kit (Ambion, Austin, TX), and translated in vitro using the Rabbit Reticulocyte Lysate System in the presence or absence of 1 μCi/ml [35S]methionine (1,200 Ci/mmol, Amersham Pharmacia Biotech) for 60 min at 30 °C, and stopped by incubation with 1 μg of cycloheximide for 5 min on ice. For trypsin digests, samples were digested with 2.4 μg of trypsin for 10 min at 25 °C, and the reactions stopped by addition of protease inhibitors. Samples exposed to detergent were made to 1% Triton X-100 prior to addition of trypsin. The reactions were analyzed by SDS-PAGE, fixed, and exposed to x-ray film.

Western Blot Analysis—Forty μg of membranes were resolved by 15% SDS-PAGE, transferred to polyvinylidene difluoride membrane (Bio-Rad), and immunoblotted with antibodies directed against RCP (R82), CRLR, and RAMP1. Membranes were then washed with phosphate-buffered saline plus 1% milk with 0.04% Tween 20 (PBS-T) and incubated with 50 μg/ml rabbit anti-chicken antibody conjugated to horseradish peroxidase (Amersham Pharmacia Biotech) for 30 min. The membranes were washed with PBS-T, incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) for 5 min, and exposed to film.

RESULTS

We determined the role of RCP in CGRP-mediated signal transduction using mammalian tissue culture cells. Western blot analysis was first performed on NIH3T3 cells and COS-7 cells to determine if RCP, CRLR, and RAMP1 expression correlated with CGRP receptor function in cell culture. Surprisingly, RCP was detected in both cell lines, while CRLR and RAMP1 were limited to NIH3T3 cells (Fig. 1A). Functional CGRP receptors were detected by RCP assays only in NIH3T3 cells, correlating with expression of all three proteins (Fig. 1B). Our findings of endogenous RCP expression in COS-7 cells explains why RCP was not needed in previous experiments, where co-transfection of CRLR and RAMP1 alone resulted in functional CGRP receptors in COS-7 cells (13, 20). We screened several immortalized cell lines by Western blot, and detected RCP expression in all cell lines tested (data not shown). Our survey was not exhaustive and we do not know if this extended expression of RCP in cell lines is significant, but it is in contrast to previous in vivo studies which found RCP expression limited to distinct populations of cells in the cochlea, brain, and eye (12, 16, 21).

Since cell lines were not identified which lacked endogenous RCP expression, gain of function experiments were not feasible. Therefore, to determine the role of RCP in CGRP-mediated signal transduction, stable cell lines were constructed that expressed antisense CGRP RNA, and the signal transduction and ligand binding in the RCP-depleted cells was determined. NIH3T3 cells contain CGRP receptors (Fig. 1B) and we have determined that these CGRP receptors exhibit Type I CGRP receptor pharmacology (data not shown), consistent with the pharmacological profile of the CGRP receptor CRLR (11, 13, 22). Antisense strategies have evolved into an effective method
of inhibiting protein expression in cell culture where expression of antisense RNA results in loss of protein expression, either by increased RNA turnover or by blockage of protein translation (23, 24). RCP cDNA was constructed as described previously (16) and a plasmid expressing the RCP cDNA in the antisense orientation was transfected into NIH3T3 cells. Stable cell lines were isolated and screened first for loss of RCP protein expression by Western blot analysis, and then for CGRP-induced cAMP response. RCP protein was not detected by Western blot in the three independent antisense cell lines when 40 μg of membrane protein were loaded per lane (Fig. 2A), and this diminished RCP protein expression correlated with a reduction of CGRP-induced cAMP production to 33% of wild-type (Fig. 2B). The CGRP-induced cAMP response is not completely abolished, most likely because antisense strategies are not 100% effective, and in fact subsequent overloaded Western blots (225 μg of membrane protein per lane) did reveal low levels of residual RCP expression in the antisense cells (data not shown).

RCP might couple the CGRP receptor to downstream effector molecules in the signal transduction pathway, or it might route the receptor to the cell surface, as is the case for RAMP1. To discriminate between these possibilities, competitive CGRP binding experiments were performed on membrane fractions prepared from control NIH3T3 cells and the three RCP-antisense cell lines. As shown in Fig. 2C, the loss of RCP did not alter the IC50 for CGRP (0.42 ± 0.17 nM for control NIH3T3 cells, 0.59 ± 0.21 nM for antisense cells), indicating that loss of RCP did not change the affinity of the receptor for CGRP in the antisense NIH3T3 cells. CGRP receptor density was also not diminished between the control NIH3T3 cells and the antisense cell lines (NIH3T3 = 0.85 ± 0.05 fmol/mg, and antisense cell lines = 1.26 ± 0.095 fmol/mg). The diminution of CGRP-mediated signaling in RCP antisense cells, together with the undiminished CGRP receptor affinity and receptor density, suggests that RCP couples the CGRP receptor to the cellular signal transduction machinery, and is not involved in routing the receptor to the cell surface. Since it has been demonstrated previously that the CGRP receptor CRLR requires the chaperone RAMP1 for function, we propose that a functional CGRP receptor complex requires at least three proteins: the ligand-binding protein (CRLR), a chaperone protein to route CRLR to the cell surface (RAMP1), and a protein to couple the receptor to the cellular signal transduction pathway (RCP) (Fig. 3).

Fig. 2. RCP is required for CGRP-mediated signal transduction, but not CGRP binding in NIH3T3 cells. A, Western blot of control (untransfected) NIH3T3 cells and antisense-RCP cell lines (numbers 52, 65, and 67). Membrane fractions (40 mg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane and Western blot analysis was performed using a rabbit polyclonal antibody R82 directed against RCP. Monomer (20 kDa), dimer (42 kDa), and trimer (60 kDa) of RCP were observed. B, CGRP-mediated cAMP response in control NIH3T3 and antisense cells. C, 125I-CGRP binding in control NIH3T3 and antisense cells. Membranes were incubated with 125I-CGRP and increasing concentrations of unlabeled CGRP, and specific CGRP binding determined.
RCP Is Required for CGRP and Adrenomedullin-mediated Signaling

Fig. 3. Model for functional CGRP receptor, including ligand-binding protein (CRLR), coupling protein (RCP), and chaperone protein (RAMP1/2).

Fig. 4. RCP is required for signal transduction at adrenomedullin, but not adenosine (A2b) or β-adrenergic receptors in NIH3T3 cells. A, adrenomedullin-induced cAMP response. B, 5'-N-ethylcarboxamidoadenosine (NECA) (A2b receptor agonist)-induced cAMP response. C, isoproterenol (β2 adrenergic receptor agonist)-induced cAMP response.

elucidate the mechanism of RCP action, an in vitro protease protection assay was used to determine whether RCP is extracellular or intracellular. Extracellular proteins are usually transported out of the cell by co-translational insertion into the endoplasmic reticulum, followed by vesicular transport to the Golgi apparatus, and from there to the cell surface by secretory vesicles. In the protease protection assay, proteins are translated in vitro in the presence of microsomes (endoplasmic reticulum/Golgi fractions), and secreted proteins are inserted into the microsomes and therefore protected from digestion by protease (27). Hence, if RCP is an extracellular protein, it should be protected from protease degradation, and if RCP is intracellular it should be degraded in this assay. Human RCP cDNA (GenBank number AF073792) was transcribed and translated in vitro in the presence or absence of microsomal membranes. Protein translation was stopped by addition of cycloheximide, trypsin was added, and samples separated by SDS-PAGE. As shown in Fig. 6A (top panel), the sensitivity of RCP to digestion from trypsin (second lane) was not diminished by addition of microsomes (fourth lane), indicating that RCP was not incorporated into microsomes and is thus intracellular. In contrast, the yeast α-mating factor (a secreted protein) (Fig. 6A, bottom panel) was protected from protease digestion (fourth lane), and higher molecular weight glycosylated forms were observed indicating incorporation of yeast α-mating factor protein into microsomes. The low molecular weight form of yeast α-mating factor which was translated independent of the addition of microsomes (first and fourth lanes) was not protected from trypsin digestion, indicating that it had not yet been incorporated into microsomes (lane 4). This protection from trypsin digestion was sensitive to detergent, indicating that a lipid membrane mediated the protection (lane 5).

The results from Figs. 1 and 6 suggest that RCP is an intracellular membrane-associated protein. Peripheral membrane proteins attached to the plasma membrane via weak ionic interactions can often be removed by increased salt concentration or increased pH; whereas membrane-spanning proteins or proteins attached to membranes by covalent interactions such as lipid attachments remain in the membrane fraction. To determine how RCP is attached to the cell membrane, membrane fractions from NIH3T3 cells were incubated with 0.1 M Na2CO3, centrifuged, and analyzed by Western blot to determine which fraction(s) contained RCP. As shown in Fig. 6B, RCP was removed from the membrane fraction after incubation with 0.1 M Na2CO3, suggesting that it was a peripheral membrane protein. This result was confirmed by Triton X-114 phase extraction, which preferentially extracts peripheral proteins into the aqueous phase and integral or lipid-attached

bubellum indicating that a physiological complex exists between RCP and CRLR. The size of CRLR protein was determined by Western blot analysis on membrane fractions prepared from cerebellum (fourth lane). A protein band (molecular mass ~60–70 kDa) was seen which agreed with previously published results (13, 20). To confirm that CRLR was solubilized under the conditions used in this experiment, CRLR was immunoprecipitated from cerebellum using OA-910 (CRLR rabbit polyclonal antibody) followed by immunoblotting with OA-910. The expected size protein (~60–70 kDa) was seen in the third lane along with the cross-reactive rabbit IgG heavy chain (~50 kDa). The RCP antibody which was raised in chicken was not recognized by the anti-rabbit secondary antibody used to detect OA-910 (first lane).

If RCP is coupling the CGRP receptor the signal transduction machinery, it should be an intracellular protein. To further
must include at least three proteins in a complex: the ligand binding, membrane-spanning protein (CRLR), a chaperone (RAMP1 or RAMP2), and a coupling protein for signal transduction (RCP) (Fig. 3).

The requirement for a trio of proteins to form a functional CGRP receptor complex can account for the difficulty in identifying the CGRP receptor. The receptor (CRLR) itself was originally cloned by reverse transcription-polymerase chain reaction, but did not respond to CGRP when transfected in cell culture (9, 10). The accessory proteins RCP and RAMP1 were both cloned independently using a X. laevis oocyte expression cloning assay (12, 13), and CGRP receptor pharmacology was demonstrated by co-transfection of RAMP1 with CRLR into COS-7 cells. RCP did not need to be co-transfected for the CGRP receptor phenotype in these previous experiments because they were performed in COS-7 cells, which we have now shown to express endogenous RCP (Fig. 1). Furthermore, we directly demonstrated the role of RCP in CGRP and adrenomedullin-mediated signal transduction by making RCP-antisense NIH3T3 cells, in which loss of RCP correlated with loss of CGRP and adrenomedullin-mediated signal transduction (Figs. 2 and 4). We could detect no change in CGRP receptor affinity or density in RCP-antisense cells compared with control cells. Thus, unlike the RAMPs, RCP does not appear to be a chaperone. Instead, RCP couples the receptor (CRLR) to the cellular signal transduction machinery, and co-immunoprecipitation studies (Fig. 5) suggest that RCP directly interacts with CRLR. The nature of the coupling mediated by RCP is still unclear: RCP may facilitate CRLR activation, couple the receptor to G-proteins or effector molecules, or coordinate the receptor-effector complex in the plasma membrane. Future experiments will elucidate the nature of coupling mediated by RCP. RCP is not a generic signal transduction protein, as signal transduction at two other G protein-coupled receptors in NIH3T3 cells was unaffected by the loss of RCP (Fig. 4, B and C). Instead, RCP may be specific for CRLR or restricted to a subset of G protein-coupled receptors.

RCP represents a new class of proteins that facilitate signal transduction at G protein-coupled receptors and the correlation between RCP expression and CGRP potency in vivo suggests that such accessory proteins might be targets for therapeutic intervention. The requirement for accessory proteins may explain some of the G protein-coupled receptors for which no ligand is known. As the CGRP receptor (CRLR) requires RCP and RAMP for function, so might these orphan receptors require additional proteins for activation by their ligand. As additional accessory proteins are identified by protein-interaction screens, antisense studies will aid in assigning functions to this emerging class of proteins involved in signal transduction at G protein-coupled receptors.

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