Intermedin is a calcitonin/CGRP family peptide
acting through the CRLR/RAMP receptor complexes

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Running Title: Intermedin is a novel calcitonin/CGRP family peptide
Summary

Calcitonin, calcitonin gene-related peptide (CGRP), adrenomedullin (ADM), and amylin belong to a unique group of peptide hormones important for homeostasis in diverse tissues. Calcitonin is essential for calcium balance whereas CGRP and ADM are important for neurotransmission, and cardiovascular and respiratory regulation. Based on phylogenetic analysis, we identified intermedin as a novel member of the calcitonin/CGRP peptide family. Analysis of intermedin expression indicated that intermedin is expressed primarily in the pituitary and gastrointestinal tract. Intermedin increases cAMP production in SK-N-MC and L6 cells expressing endogenous CGRP receptors and competes with labeled CGRP for binding to its receptors in these cells. In addition, treatment of 293T cells expressing recombinant calcitonin receptor-like receptor (CRLR) and one of the three receptor activity modifying proteins (RAMPs) showed that a CRLR/RAMP receptor complex is required for intermedin signaling. In contrast to CGRP and ADM, which exhibit a preferential stimulation of CRLR when coexpressed with RAMP1 and RAMP2 or RAMP3, respectively, intermedin represents a nonselective agonist for the RAMP co-receptors. In vivo studies demonstrated that intermedin treatment leads to blood pressure reduction in both normal and spontaneously hypertensive rats via interactions with the CRLR/RAMP receptors. Furthermore, in vivo treatment in mice with intermedin leads to a suppression of gastric emptying activity and food intake. Thus, identification of intermedin as a novel member of the calcitonin/CGRP peptide family capable of signaling through the CRLR/RAMP receptor complexes provides an additional player in the regulation of peripheral tissues by CRLR, and will allow development of new therapeutic agents for pathologies associated with diverse vascular and gastrointestinal disorders.
Introduction

Originally isolated as a polypeptide hormone essential for calcium balance (1,2), calcitonin belongs to a group of peptide hormones including αCGRP, βCGRP, adrenomedullin (ADM), and amylin (3). Among these tissue-specific peptides, ADM and CGRP are important endocrine and neurocrine integrators of homeostasis in the vascular and respiratory systems whereas amylin is essential for optimal glucose metabolism. The biological actions of these peptides are mediated via binding to two closely related type II G protein-coupled receptors (GPCRs), the calcitonin receptor and the calcitonin receptor-like receptor (CRLR)(4,5). Although the calcitonin receptor is the main mediator for calcitonin action, it also binds amylin. Recent cloning and functional studies have shown that CGRP, ADM, and to a lesser extent, amylin, interact with different combinations of CRLR and the three receptor activity modifying proteins (RAMPs)(5,6). Studies using mutant mice deficient for αCGRP, ADM, or amylin have indicated that CRLR could be important for cardiovascular morphogenesis, sensory neurotransmission, inflammatory reactions, nociceptive behavior, and glucose homeostasis (7-12). Thus, the physiological functions of the peptides in this family are determined by receptor-binding specificity and the tissue expression profiles of individual ligands.

Because the expression of CGRP and its binding sites do not overlap in the brain (13), we hypothesized the existence of additional calcitonin/CGRP family peptides. Using a phylogenetic profiling approach to analyze GenBank databases, we have identified a novel calcitonin/CGRP family peptide, intermedin, from the genomes of human and other vertebrates. Sequence analysis of the prepro-polypeptides of different family genes indicated that the sequence homology between intermedin and the paralogous peptides is restricted to the mature peptide, and that the intermedin gene evolved during early vertebrate evolution. Pharmacological analyses showed
that intermedin signals through CRLR/RAMP receptor complexes and activates the cAMP-dependent pathway in transfected cells. We have also shown that intermedin signals through the CRLR signaling system to regulate vascular and gastrointestinal functions \textit{in vivo}.
Experimental Procedures

Cloning, phylogenetic analysis, and expression profiles of human intermedin. Human intermedin was initially identified from an EST and a genomic sequence (AK024788 and AL096767) and its identity was verified by PCR amplification using a human Marathon-ready pituitary cDNA library (Clontech, Inc., Palo Alto, CA). For analysis of intermedin mRNAs in the human digestive system, normalized first strand cDNA preparations were obtained from Clontech, Inc. The putative intermedin peptides from fish were deduced based on a zebrafish EST sequence (AW421384) and puffer fish genomic sequences (Fugu rubripes Scaffold_1011), respectively. The rat and mouse intermedin sequences were deduced based on EST BQ192607 and BG918210, respectively. Putative puffer fish αCGRP, βCGRP, ADM, and amylin were deduced based on puffer fish sequences Scaffold_9445, Scaffold_6549, JGI_28042, and JGI_8403, respectively (http://www.jgi.doe.gov/fugu/index.html). The BLOCK MAKER program (http://blocks.fhcrc.org) was used to align the mature peptides from different species. Phylogenetic analysis was carried out using a routine in ClustalW (http://blocks.fhcrc.org/blocks). The consensus secondary structure of calcitonin/CGRP family peptides was predicted using the Network Protein Sequence Analysis server (http://pbil.ibcp.fr/). For Northern blotting analysis of intermedin expression, pituitary RNAs were extracted from pituitary glands obtained from male Sprague-Dawley rats. Following extraction using TriZol solution, total RNA was resolved using formaldehyde agarose gels and hybridized with a 32P-labeled rat intermedin cDNA probe. The x-ray film was exposed at -80C for one week with intensifying screens.
Peptide synthesis. Intermedin-related peptides were synthesized based on the solid phase fluorenylmethoxycarbonyl protocol and analyzed by reverse phase HPLC with Vydac C18 analytical column and mass spectrometry using a MALDI-TOF Voyager-DE RP Workstation. Synthetic ADM, βCGRP, and related peptides were obtained from Sigma-Aldrich Corp. (St. Louis, MO), AnaSpec, Inc. (San Jose, CA), and Bachem (Torrance, CA). Radiolabeled $^{125}$I-CGRP (2,000 Ci/mmole) was from Amersham Pharmacia (Arlington Heights, IL). Stocks of different hormones were prepared in distilled water and diluted in culture medium.

Immunoanalysis. Rabbit anti-intermedin antibodies were generated using synthetic peptides corresponding to residues 28-47, MGPAGRQDSAPVDPSSPHSY, of human intermedin (Strategic Biosolutions, Ramona, CA). This peptide antigen was selected based on the high sequence identity (85%) found in this region of human and rodent intermedins and the negligible similarity with other family peptides. The intermedin peptide was conjugated to the keyhole limpet hemocyanin using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride before immunization. Antibodies were purified using antigen-conjugated affinity columns. In immunoblot analysis, the anti-human intermedin antibody cross-reacted with synthetic intermedin counterparts from different vertebrates but not paralogous peptides including calcitonin, CGRP, ADM, and amylin. For immunohistochemical analysis, tissues were obtained from adult rats, mice, and bullfrogs, and analyzed as described (14). To demonstrate that intermedin transcript encodes the predicted intermedin mature peptide, full-length human intermedin cDNA was subcloned into the pcDNA3.1 expression vector. For Western blotting analysis of intermedin in culture media, 293T cells were transfected with the intermedin expression vector using the calcium phosphate precipitation method. Forty-eight hours after
transfection, serum-free culture media were harvested and concentrated using a Centricon 3 column. After concentration, the supernatant was boiled for 5 min in denaturing buffer with 100 mM dithiothreitol before SDS-PAGE and Western blotting analysis using anti-intermedin antibodies.

_Stimulation of cAMP production in SK-N-MC and L6 cells by intermedin and related peptides._

Human neuroblastoma SK-N-MC and rat L6 skeletal myoblast cells expressing endogenous CRLR were obtained from American Type Culture Collection. To estimate adenylyl cyclase activation, SK-N-MC and L6 cells (2x10^5 viable cells/well) were plated in 24-well culture dishes in DMEM/F12 medium one day before treatment. Following 2 h incubation in serum-free DMEM/F12 medium, cells were treated with testing reagents for 30 min in medium containing 0.1% BSA and 2.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Corp.) to prevent hydrolysis of cAMP by phosphodiesterases. Following treatment, cells were lysed and cAMP content determined by a specific radioimmunoassay (15).

_Activation of CRLR/RAMP receptor complexes by intermedin in transfected 293T cells._ To study the interaction between intermedin and the CRLR/RAMP receptor complexes, we cloned human CRLR, RAMP1, RAMP2, and RAMP3 (accession numbers NP_005786, NP_005846, NP_005845, and O60896, respectively) cDNAs by PCR from human Marathon-ready cDNA libraries using two sets of primers flanking the full-length coding sequences of each gene. Each cDNA was verified by DNA sequencing and subcloned into the expression vector pcDNA3.1. To allow the detection of cell surface expression of these proteins, CRLR and RAMP proteins were
tagged at the N-terminus of the mature protein with a FLAG epitope. Because it has been shown that epitope tagging affects the signaling by RAMP1 protein (16), we used the wild type RAMP1 construct for the analysis of intermedin signaling. HEK293T cells were maintained in 35-mm culture dishes in DMEM/Ham’s F-12 (Life Technologies, Inc.) supplemented with 10% FBS, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine. The cells were co-transfected with 10 µg CRLR and/or 10 µg RAMP expression plasmid using the calcium phosphate precipitation method. Forty-eight hours after transfection, cells were washed twice with Dulbecco’s PBS (D-PBS), harvested from culture dishes, and centrifuged at 400 x g for 5 min. To determine the level of expression of CRLR and RAMP on the cell surface, the resuspended cells (2 × 10^6/tube) were incubated with the FLAG M1 antibody (50 mg/ml) (Sigma-Aldrich Corp.) in Tris-buffered saline (pH 7.4) containing 5 mg/ml bovine serum albumin and 2 mM CaCl₂ (assay buffer) for 4 h at room temperature in siliconized tubes. Cells were then washed twice with 1 ml of assay buffer after centrifugation at 14,000 × g for 15 sec. The horseradish peroxidase-conjugated secondary antibody (sheep anti-mouse IgG) was added to the resuspended cell pellets and incubated for 1 h at room temperature. Cells were washed twice with 1 ml of assay buffer before determination of horseradish peroxidase activity in cell pellets using the ECL reagents (Amersham Bioscience) and a Lumimark microplate reader (Bio-Rad, Inc.). Background binding was determined by adding excess amounts of the synthetic FLAG peptide (Sigma-Aldrich Corp.) at a concentration of 100 µg/ml.

For the assay of adenylyl cyclase activation in transfected cells, cells (2 x 10^5/ml) were placed in 24-well tissue culture plates (Corning, Inc. Corning, NY) and preincubated at 37C for 30 min in the presence of 2.5 mM IBMX before hormonal treatment for 4 h.
Receptor-binding assay. Ligand-binding assays were done in siliconized microfuge tubes at 37°C for 2 h. Intact SK-N-MC and L6 cells were resuspended in the binding buffer (20 mM Tris-HCl, pH 7.4, 2 mM MgCl2, and 0.1% BSA) with 0.06 µg of 125I-CGRP and various concentrations of nonradioactive peptides. After a 2 h incubation at 37°C, the cell-associated ligand was estimated (15). Radioactivity was determined using a γ-counter (EG&G Wallace, Gaithersburg, MD).

Effects of intermedin on blood pressure and heart rate in normal and hypertensive rats. Blood pressure measurements in conscious male Sprague-Dawley rats and spontaneously hypertensive rats (SHR) (7-9 weeks of age) were made in animals preadapted to the measurement procedure. Indirect systolic pressure was determined by a programmable NIBP system using the tail-cuff method (Columbus Instruments, Columbus, OH). Following attachment of the pressure transducer, rats were left undisturbed for 10 min before baseline measurements that spanned a 15-min interval. Following baseline measurements, rats were injected intraperitoneally with varying doses of hormones. Blood pressure and heart rate were monitored for 40 min at 20-sec intervals. Changes in blood pressure were calculated as the average of thirty measurements performed within each 10-min interval.

Effects of intermedin on gastric emptying activity. Eight-week-old C57/BL6 male mice deprived of food for 20 h were given food pellets for 90 min before intraperitoneal injection with different hormones or saline. After treatment, mice were deprived of food again and killed 90 min later. The stomach was excised at the pylorus and cardia before weighing. Gastric emptying was
calculated by comparing the stomach weight of treated mice to the stomach weight of control
mice killed at the time of hormone injection.

_Analysis of ingestive behavior._ Eight-week-old C57/BL6 male mice were housed individually in
a regulated environment. Before intraperitoneal injection with testing reagents, mice were
derived of food for 20 h with free access to water. Food intake was measured by placing
preweighed pellets in the cage and weighing uneaten pellets at 1, 2, and 4 h after treatment.

_Statistical analysis._ Differences between treatment groups were analyzed using ANOVA and
Student’s t-test.
Results

*Intermedin as a calcitonin/CGRP family peptide.* We searched GenBank databases for sequence motifs with unique primary and secondary structures shared by all calcitonin/CGRP family peptides using a phylogenetic profiling approach that has allowed the identification of novel CRH family peptides (15). Candidate sequences were screened for the presence of proteolytic cleavage sites flanking the putative mature region of the precursor proteins. Based on these criteria, we have identified intermedin genes from mammals and teleosts including zebrafish and a Japanese puffer fish (*Takifugu rubripes*). Human intermedin encodes a prepro-protein of 148 amino acids with a signal peptide for secretion at the N-terminus (Fig. 1A). Although the overall amino acid sequence of intermedin showed no similarity to known proteins, a stretch of 47 residues at the C-terminus is flanked by dibasic proteolytic cleavage sites at the N-terminus and an alpha-amidation donor residue at the C-terminus. The putative mature region of intermedin shares approximately 28% sequence identity with ADM and <20% with CGRP (Fig. 1B). Importantly, the predicted mature region adopted an amino-terminal disulfide-bonded loop leading into an alpha-helix followed by a disordered structure that is shared by all calcitonin/CGRP family peptides (Fig. 1B). Furthermore, sequence alignment of intermedin precursors from mammals and teleosts indicated that sequence conservation in orthologous intermedins is restricted to the mature region. The mature intermedins of human and fish share a >60% similarity whereas human and rodent intermedins are 87% identical (Fig. 1B). In addition, the mouse and rat intermedin peptides appeared to differ by only one amino acid. Furthermore, analysis of orthologous intermedin indicated that the position of N-terminal dibasic cleavage sites varied by a few amino acids among different species whereas an arginine residue seven amino acids downstream of the dibasic cleavage motif of human intermedin is conserved in all
species, suggesting that the mature intermedin from human and other species could be a 40-amino-acid peptide. On the basis of these sequence analyses, we predicted that a 47-amino-acid mature peptide (intermedin-long or IMDL) and a shorter 40-amino-acid intermedin (intermedin-short or IMDS) could be generated by proteolytic cleavage at the N-terminal proximate basic residues followed by an amidated C-terminus. Because the putative prepro-region of intermedin from diverse vertebrates is not conserved, intermedin is unlikely to encode additional active peptides such as the proADM N-terminal 20 peptide (PAMP) found in the ADM precursor (17).

Phylogenetic analysis of twelve CGRP family peptides from fish and mammals suggested an ancient evolution for three subgroups of these peptide hormones with mammalian and teleost intermedins clustered in a separate branch with ADM and CGRP (Fig. 1C). Thus, intermedin and other family peptides evolved before the emergence of modern teleosts and tetrapods. Genomic analysis showed that intermedin is located on the distal arm of human chromosome 22q13 and syntenic mouse chromosome 15. In both human and mouse genomes, intermedin neighbors an aldehyde reductase-like gene. In contrast, all other calcitonin/CGRP family genes cluster on human chromosomes 11 and 12.

*Intermedin activates the cAMP-dependent pathway in SK-N-MC and L6 cells via the CGRP receptor.* Pairwise sequence comparison and phylogenetic tree building based on all GPCR sequences indicated that intermedin is closest to ADM and CGRP whereas no orphan GPCR shares a close relatedness to CRLR, the receptor for ADM and CGRP. Thus, CRLR is a candidate receptor for intermedin. To test this hypothesis, we treated human neuroblastoma-derived SK-N-MC cells and rat L6 skeletal myoblast cells, known to express different levels of CRLR and RAMPs (18), with synthetic intermedin peptides, and then monitored cAMP
production. As shown in Figs. 2A and 2B, treatment with amidated long intermedin peptide (amino acid 1-47, IMDL) or short intermedin (amino acid 8-47, IMDS) resulted in dose-dependent increases of cAMP production in both cell lines. The observed activation is specific as treatments with a nonamidated form of intermedin, a truncated amidated intermedin fragment (intermedin17-47, IMD17-47), or a 31-amino-acid peptide from the prepro-region of human intermedin (prointermedin55-85) have no effect in either cell line (data not shown), suggesting that α-amidation and residues 8-16 of intermedin are important for intermedin bioactivity.

Consistent with earlier reports (5,18,19), both ADM and βCGRP also stimulated cAMP production in these cell lines (Figs. 2A and 2B). Of importance, the stimulatory effect of intermedin was suppressed by cotreatment with a CGRP receptor antagonist, CGRP8-37, in L6 cells (Fig. 2C) demonstrating that intermedin activates the cAMP-dependent pathway via the CGRP receptor (20). To further characterize the specific action of intermedin on cAMP production, L6 cells were cotreated with a putative intermedin C-terminal receptor-binding domain, IMD17-47, or an anti-intermedin polyclonal antibody. As shown in Fig. 2D, IMD17-47 was found to be a functional antagonist of intermedin action, consistent with the observed antagonistic effect of N-terminally truncated CGRP8-37 (Fig. 2C)(20). In addition, cotreatment with the anti-intermedin antibody blocked the stimulatory effect of intermedin whereas cotreatment with an antibody raised against the unrelated stresscopin-related peptide (SRP)/urocortin II had no effect (Fig. 2D).

To establish a direct interaction between intermedin and CRLR, we used iodinated CGRP as the radioligand for receptor-binding assays. As shown in Figs. 2E and 2F, IMDL and IMDS displaced \(^{125}\)I-CGRP binding to the SK-N-MC and L6 cells dose-dependently.
Intermedin is a nonselective agonist for CRLR/RAMP receptor complexes. CGRP and adrenomedullin mediate their action through the CRLR/RAMP complexes consisting of CRLR and one of the three RAMP polypeptides. To investigate the role of CRLR/RAMP receptor complexes in intermedin signaling, we treated 293T cells expressing different combinations of recombinant CRLR and/or RAMP proteins with intermedin and related peptides. As shown in Fig. 3A, treatment of intermedin, CGRP, or ADM has no effect on the cAMP production in 293T cells expressing CRLR alone whereas calcitonin increases cAMP dose-dependently via the endogenous calcitonin receptor. In contrast, intermedin stimulates cAMP production in cells expressing different CRLR/RAMP receptor complexes dose-dependently (Figs. 3B-3D).

Consistent with earlier studies, CGRP and ADM exhibit a preferential stimulation of CRLR when coexpressed with RAMP1 and RAMP2 or RAMP3, respectively. As compared to CGRP, intermedin exhibits a greater potency in the stimulation of cAMP production in cells expressing CRLR/RAMP3, but has lower activity on the CRLR/RAMP1 complex. In contrast, intermedin has a lower potency on the activation of both CRLR/RAMP2 and CRLR/RAMP3 as compared to adrenomedullin. Thus, the overall rank of potency for the stimulation of CRLR/RAMP1, CRLR/RAMP2, and CRLR/RAMP3 are CGRP>IMD=ADM, ADM>IMD=CGRP, and ADM>IMD>CGRP, respectively. Further, consistent with earlier reports, the expression of CRLR/RAMP receptors on the cell surface of transfected cells was found to be increased synergistically by coexpressing CRLR and RAMP proteins (Fig. 3E).

Intermedin expression in the pituitary and stomach. Initial RT-PCR analysis showed that the intermedin transcript is expressed in the pituitary and stomach. Northern blotting analysis of rat pituitary RNA showed that two specific intermedin transcripts of approximately 5 and 2.5 kb are
present in the pituitary (Fig. 4A). To further characterize the expression profile of intermedin, four independent antibodies were developed using a C-terminal 20-amino-acid intermedin peptide (IMD28-47). As shown in Fig. 4B, the anti-intermedin antibody (C2411-2) is specific for intermedin and shows no cross-reaction with related peptides including calcitonin, CGRP, ADM, or amylin. Using the specific anti-intermedin antibody, immunohistochemical analysis of more than twenty different mouse tissues confirmed intermedin expression in the pituitary and the stomach. As shown in Figs. 4C (X100) and 4D (X200), intermedin is expressed mainly in the intermediate lobe of the pituitary with sporadic signals in the anterior lobe. In contrast, negative controls using preimmune serum or anti-intermedin antibodies presaturated with the intermedin antigen showed no specific signals (Figs. 4E and 4F). Likewise, immunohistochemical analysis of pituitary sections from rats and bullfrogs showed that intermedin expression is restricted to the intermediate and anterior lobes of pituitary (Figs. 4G and 4H). Because melanin-stimulating hormone (MSH) has a similar expression pattern in pituitary (Fig. 4I, anti-MSH staining), we tested whether the anti-intermedin antibody cross-reacts with the MSH peptide. As shown in Fig. 4J, the specific staining of intermedin in the pituitary was not abolished by preincubating with an MSH peptide. To further demonstrate that the intermedin mRNA encodes the predicted mature intermedin peptide, a human intermedin cDNA was subcloned in the eukaryotic expression vector pcDNA3.1, and the expression of intermedin peptide from this construct was investigated using transfected 293T cells. Western blotting analysis of concentrated culture media showed that cells transfected with the intermedin expression vector secrete an approximately 5 kDa mature intermedin peptide into the culture media whereas culture media from cells transfected with the empty vector display no signal (Fig. 4K).
To characterize the expression of intermedin in the gastrointestinal tract, a panel of human cDNA from the gastrointestinal tract was analyzed by PCR. As shown in Fig. 5A (upper panel, 1 ng cDNA template/tube), the expression of the intermedin transcript could be detected in the esophagus, stomach, jejunum, ileum, ileocecum, ascending colon, transverse colon, descending colon, and rectum. PCR analysis using a lower amount (10 pg/tube) of cDNA templates showed that the expression of the intermedin transcript is greater in the stomach and jejunum (Fig. 5A, lower panel). Further, immunohistochemcal staining showed that intermedin is found primarily in the muscularis mucosae layer of stomach (Fig. 5B) and the signal is abolished by presaturation with the intermedin antigen (Fig. 5C).

Systemic hypotensive action of intermedin. Because the related ADM is one of the most potent vasodilators (5) and the pituitary-derived intermedin could be released into systemic circulation to act on diverse peripheral tissues, we tested the effect of intermedin on blood pressure regulation in normal rats and SHR using a noninvasive monitoring approach. As shown in Fig. 6A, intraperitoneal administration of IMDL or IMDS dose-dependently suppressed blood pressure in normal Sprague-Dawley rats, similar to that induced by ADM. In addition, treatment of IMDL or IMDS also increased heart rate as found for ADM (Fig. 6B). In contrast, administration of the truncated IMD17-47 fragment (Fig. 6C) or the prointermedin55-85 peptide (data not shown) had no effect on blood pressure regulation. Because intermedin signals through CRLR/RAMP receptor complexes, the ability of a CGRP receptor antagonist CGRP8-37 to block the actions of intermedin was also studied. As shown in Fig. 6C, treatment with 20-fold excess CGRP8-37 significantly decreased the hypotensive effects of IMDL. Likewise, cotreatment with the putative intermedin receptor-binding domain fragment IMD17-47 blocked
the hypotensive effects of IMDL. In addition, we have studied the hypotensive effect of intermedin in spontaneous hypertensive rats (SHR). Similar to normal rats, IMDL treatment reduced blood pressure in SHR and the hypotensive effects of IMDL were abolished by cotreatment with CGRP8-37 (Fig. 6D). In contrast, cotreatment with the low affinity ADM22-52 fragment had minimal effect (21). Thus, intermedin is a specific ligand for the vascular CRLR/RAMP signaling system and could be important in the mediation of vascular responses for homeostasis.

*Intermedin suppresses food intake and gastric emptying.* Earlier studies have shown that both CGRP and ADM have potent anorexic effects (22) and could mediate actions through central or peripheral CRLR/RAMP systems. To examine whether intermedin has a role in anorexia regulation, we studied the ability of intermedin to regulate feeding behavior based on cumulative food intake in fasted mice. Intraperitoneal injection with IMDL, IMDS, ADM, or a type II CRH receptor-selective agonist SRP/urocortin II, decreased food intake in fasted mice (15,23)(Fig. 7). Because intermedin is specifically expressed in the muscularis mucosae layer of stomach, it could have a role in gastrointestinal functions. We therefore studied the ability of intermedin to regulate gastric emptying activity in mice. As shown in Fig. 8, intraperitoneal administration of intermedin suppressed gastric emptying activity, similar to the treatment with a known gastric emptying suppression peptide, SRP/urocortin II (15,23). Likewise, treatment with ADM also suppressed the gastric emptying activity, but with a lower potency. Thus, intermedin could mediate anorexic responses through the regulation of gastrointestinal motility (22,24).
Discussion

We have used a genomic approach to study novel polypeptide ligands and receptors based on the evolutionary conservation of polypeptides (14,15,25,26). Based on the analysis of the evolution of calcitonin/CGRP family ligands from diverse vertebrates, we have identified a novel family peptide, intermedin, that is expressed in the pituitary and the digestive tract. Studies using 293T cells expressing recombinant CRLR and RAMP proteins demonstrated that intermedin is a bioactive peptide and activates the Gs of the G protein family through CRLR/RAMP receptor complexes. In contrast to CGRP and ADM, which exhibit a preferential stimulation of CRLR when coexpressed with RAMP1 and RAMP2 or RAMP3, respectively, intermedin represents a nonselective agonist for the three CRLR/RAMP receptor complexes.

Since the discovery of calcitonin in 1960s, the calcitonin/CGRP family peptides have been studied extensively. As a result of gene duplication and functional divergence, this group of peptide hormones acts on diverse systems. Coupled with two closely related GPCRs and three unique RAMPs that transport receptors to the cell surface, a complex ligand-receptor signaling system operates in diverse vertebrates (5). Calcitonin, CGRP, ADM, and amylin are expressed in a tissue-specific manner with the highest expression in the thyroid C cell, central nervous system, adrenal, and islet B cells, respectively. Although it has been recently established that the signaling by CGRP, ADM, and amylin is unique among peptide hormones and requires the formation of a receptor/RAMP complex, the exact role of these peptides and their cognate receptors in different physiologies remains to be investigated. The present discovery of intermedin as a calcitonin/CGRP family peptide highly expressed in the pituitary and the digestive tract provides a new ligand for peripheral regulation mediated by the CRLR/RAMP system. As a first step for defining the role of CRLR/RAMP receptor complexes in intermedin...
signaling, we investigated the activation of CRLR/RAMP receptor complexes in transfected 293T cells, and demonstrated that RAMP is required for mediating intermedin action through CRLR. Of interest, intermedin exhibited a receptor-activation profile distinct from that of CGRP or ADM, suggesting that intermedin could be important for select CRLR/RAMP-mediated physiological processes. It has been shown that the receptor-activation profiles of CGRP and ADM in native tissues are affected by endogenous RAMPs present in different systems. Future studies on the interaction between intermedin and CRLR/RAMP receptor complexes in different cell types and native tissues are needed to clarify the importance of different RAMPs in intermedin physiology. Furthermore, it has been demonstrated that even though CGRP and ADM overlap in receptor interaction, each of these peptides apparently binds to unique binding pockets (5); therefore, future studies on the structural-functional relationship of intermedin and related peptides are essential for the characterization of the CRLR/RAMP-associated signaling system.

Among calcitonin/CGRP family peptides, ADM is mainly characterized as a hypotensive hormone (27-29) whereas CGRP is important for sensory neurotransmission (3,9-11,13,30,31). In addition, ADM inhibits bronchial constriction and acts as a neurohormone to inhibit water drinking and salt appetite (27,32,33). Studies using mutant mice suggested that ADM is indispensable for vascular morphogenesis during embryonic development (7,8) whereas αCGRP is important for the modulation of sympathetic activity and inflammatory reactions (9-11). Therefore, the CRLR in different tissues could mediate the actions of multiple paralogous ligands, and the physiological role of this receptor is partly dependent on activating ligands derived from neighboring cells and/or general circulation. Because intermedin interacts with CRLR/RAMP receptor complexes, the known receptors for CGRP and ADM, intermedin could regulate diverse physiological functions that have been attributed to ADM or CGRP. As
demonstrated in the present study, intermedin decreases blood pressure in both normal rats and SHR as effectively as the better characterized ADM and CGRP, suggesting that intermedin could regulate vasculature homeostasis (27). Immunohistochemistry studies have shown that CRLR and RAMPs are found in the entire vasculature and the expression of CRLR is mainly in the endothelial layer (34), therefore, intermedin and related peptides decrease blood pressure via the activation of CRLR/RAMP receptors in the vascular endothelial cells. Concomitant with a hypotensive effect, intermedin treatment also increases heart rate. The increase of heart rate by intermedin and related peptides could be a reflex response to the hypotensive effect; however, the exact mechanisms remained to be investigated because the CRLR gene has been shown to be expressed in cardiac myocytes (35) in addition to the cardiac vasculature (34,36). Further, intermedin could have cardioprotective and antibronchial constriction activities that are important for the regulation of cardiac and respiratory homeostasis (27,37). Because intermedin is not detected in proximity to the vasculature system using immunohistochemical analysis, further studies are needed to reveal whether intermedin represents an endocrine hormone involved in the regulation of cardiac and vasculature systems.

Earlier studies on CGRP and ADM have shown that these peptides and the CRLR signaling system play an important role in gastrointestinal functions including motility and secretions from stomach and colon (38,39). Similar to earlier studies on CGRP and ADM, exogenous intermedin administration was found to exhibit an anorexic effect and suppress stomach emptying responses in mice. These data suggested that intermedin could have roles in the regulation of energy balance via a paracrine mechanism; however, it is possible that the observed effect on feeding behavior is secondary to alterations in gastric motility. Because intermedin is expressed in multiple gastrointestinal tissues, intermedin could have additional
roles in the gastrointestinal system that remain to be characterized. In support of this view, it has been shown that CRLR is expressed in columnar cells lining the secretory ducts of the parotid gland and in capillaries and venules of the esophagus (34).

The observation that intermedin is expressed in the anterior and intermediate lobes of the pituitary pointed to a potential role for intermedin and the CRLR/RAMP signaling system in the regulation of pituitary hormone secretion. Although the role of intermedin in the regulation of pituitary hormone secretion has not been examined specifically in the present study, earlier studies on ADM have shown that administration of ADM increases circulating prolactin levels in humans (40,41). Therefore, it is possible that intermedin may play a role in the regulation of pituitary functions. Further studies on the exact expression pattern of intermedin in the pituitary and other tissues during development are important for the understanding of intermedin physiology in pituitary and other tissues.

Earlier studies on the evolution of peptide hormones have shown that selection pressure has favored the conservation of functionally important or mature regions of polypeptide hormone precursors. The finding that only the C-terminal end of the intermedin precursor was conserved during evolution suggested that the C-terminal sequences of the intermedin precursors represent the mature peptide, and further strengthened the theory that sequence conservation among species provides important information on the functional characteristics of gene sequences. Of interest, comparative sequence studies of intermedin precursors from different vertebrates showed that the N-terminal cleavage site of putative mature intermedins vary in position whereas a downstream arginine residue is completely conserved in all species studied. These data indicate that mature intermedin from diverse species could be of varying lengths and a shorter human intermedin (e.g. IMDS) could be generated after posttranslational processing at the downstream
basic residue. Future studies on human samples are necessary to reveal the exact mature form(s) of human intermedin. Furthermore, the recent availability of multiple sequenced vertebrate genomes would allow the identification and characterization of additional peptide hormones on a global scale based on the genomic profiling approach we used to identify intermedin and other novel peptide hormones (15).

In conclusion, we have identified and characterized a novel calcitonin/CGRP family gene and demonstrated that encoded intermedin peptides are biologically active in diverse \textit{in vitro} and \textit{in vivo} CRLR/RAMP assays. Therefore, intermedin is a physiological regulator of gastrointestinal, cardiovascular, and other bioactivities mediated by the CRLR/RAMP receptor complexes. Although the four mammalian CGRP-related peptide hormones, \(\alpha\)CGRP, \(\beta\)CGRP, ADM, and intermedin are capable of interacting with CRLR, optimal regulation by this GPCR signaling pathway likely depends on an integrated release of different endocrine/paracrine ligands in a tissue-specific and time-coordinated manner. Future studies on tissue distribution and endogenous fluctuation of intermedin under normal or pathological conditions are important to formulate pharmacological therapies for diverse pathological conditions in cardiovascular, pulmonary, gastrointestinal, and neuroendocrine systems.
Acknowledgements

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References


Figure Legends

Fig. 1. Cloning of intermedin and elucidation of its identity.  

A. The human intermedin gene encodes a 148-amino-acid ORF with a 24-amino-acid signal peptide for secretion at the N-terminus. Amino acid numbers are on the right and the stop codon is marked with an asterisk. The putative mature peptide is underlined whereas the N-terminal signal peptide for secretion is lightly shaded. The ATG start site and the putative C-terminal amidation donor residue are in bold letters. The putative basic cleavage sites are highlighted by a dark background.  

B. Comparison of CGRP-related peptides (αCGRP, βCGRP, amylin, ADM, and intermedin (IMD)) from mammals and fish. Sequence alignment of the precursors of these peptides from human indicated the sequence homology between intermedin and paralogous peptides is restricted to the mature peptide and no similarity was found in the putative prepro-region. The two intermedins from puffer fish (Takifugu rubripes) are indicated as IMD1 and IMD2, respectively. The putative secondary structures of mature CGRP peptides are indicated above the alignment whereas the putative secondary structures of mature intermedins are shown below the alignment. In addition, the predicted IMDL and IMDS intermedin peptides are indicated by dot lines underneath the alignment. Random coil, curved line; extended strand, round cylinder; helix, wavy banner. The two cysteines and one neighboring threonine residue shared by all aligned peptides are enclosed by lines. Residues shared by ADM and intermedin from different vertebrates are indicated by bold asterisks. Residues shared by highly conserved orthologous sequences of each gene are lightly shaded. The N-terminal arginine residue found in all intermedins from different species are shown in bold Rs. h: human; m: mouse; r: rat; p: puffer fish; z: zebrafish.  

C. The phylogenetic relationship among twelve representative CGRP-related peptides. h: human; m: mouse; p: puffer fish.
Fig. 2. Intermedin shares receptors with CGRP and ADM. A. and B. Synthetic intermedin peptides (IMDL and IMDS) stimulate cAMP production in human neuroblastoma SK-N-MC cells (A) and rat L6 skeletal myoblast cells (B). No stimulation by a nonamidated form of intermedin, a truncated amidated intermedin fragment (intermedin17-47, IMD17-47), or a 31-amino-acid peptide from the prepro-region of intermedin (prointermedin55-85, proIMD55-85) was observed (data not shown). Data are mean ± s.e.m. (N=4). C. Blockage of the stimulatory effect of intermedin on cAMP production by a CGRP receptor antagonist, CGRP8-37, in L6 cells. D. Blockage of the stimulatory effect of intermedin by IMD17-47 (1 μM) and the anti-intermedin antibody (anti-IMD Ab) in L6 cells. Data are mean ± s.e.m. (N=4). No effect was observed by cotreatment with an anti-SRP/urocortin II antibody (anti-SRP Ab). *, significantly different from controls (P < 0.05). E. and F. Competitive displacement by unlabeled intermedin and related peptides of 125I-CGRP bound to SK-N-MC (E) or L6 (F) cells. Data are mean ± s.e.m. (N=3).

Fig. 3. Intermedin activates recombinant CRLR/RAMP receptor complexes in transiently transfected 293T cells. A-D. Treatment of 293T cells transiently transfected with an empty expression vector or a CRLR expression vector with intermedin, CGRP, or ADM has no effect on the whole cell cAMP production (A). In contrast, calcitonin increases cAMP production dose-dependently through the endogenous calcitonin receptor. Unlike cells expressing CRLR solely, treatment of intermedin increases cAMP production in cells expressing CRLR with RAMP1 (B), RAMP2(C), or RAMP3 (D). Likewise, treatment of CGRP or ADM stimulates cAMP production in cells expressing CRLR/RAMP receptor complexes with different potency (Figs.
Indirect binding analysis of cell surface expression of CRLR and RAMP proteins using horseradish peroxidase-conjugated sheep anti-mouse antibodies and anti-FLAG epitope antibodies. Expression of FLAG epitope-tagged CRLR and RAMPs on the cell surface of transfected cells is increased by cotransfection of CRLR and RAMP expression vectors as compared to transfection with a single expression vector encoding CRLR or RAMP.

Fig. 4. Expression of intermedin in the pituitary. A. Northern blotting analysis showed that two specific intermedin transcripts are expressed in rat pituitary cells. Positions for 28S and 18S RNA are indicated by arrows. B. Western blotting analysis of synthetic peptides using an anti-intermedin antibody generated against the C-terminal twenty amino acids of human intermedin, MGPAGRQDSAPVDPSPHSY. The anti-intermedin antibody is specific for intermedin and shows no cross-reaction with CGRP, calcitonin, ADM, or amylin. Molecular weight markers are shown on the left and specific bands are indicated by arrows. C-F. Immunohistochemical staining of mouse pituitary sections using the anti-intermedin antibody (C, X100; D, X200), preimmune rabbit serum (E), or anti-intermedin antibody presaturated with the intermedin ligand (F). Sections incubated with preimmune serum (E) or antibodies presaturated with the intermedin peptide antigen (F) showed negligible signals. G and H. Immunohistochemical analysis of intermedin expression in pituitary sections of rat (G) and bullfrog (H). I and J. Immunohistochemical staining of mouse pituitary sections using an anti-melanin-stimulating hormone (MSH) antibody (I) or the anti-intermedin antibody presaturated with an MSH peptide (J). Specific signals are indicated by arrows. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. K. Western blotting analysis of concentrated culture media from 293T cells transfected with an intermedin expression vector. The anti-intermedin antibody detected an
approximately 5 kDa mature intermedin peptide in culture media whereas media from cells transfected with the empty vector displayed no signal. Specific intermedin signals are indicated by an arrow. Positive signals from the synthetic intermedin peptide are shown on left lanes.

**Fig. 5. Expression of intermedin in digestive tissues.** A. For the analysis of intermedin mRNAs in the human digestive system, normalized first strand cDNA preparations from human esophagus, stomach, jejunum, duodenum, ileum, ileocecum, cecum, ascending colon, descending colon, transverse colon, and rectum (higher panel, 1 ng template/reaction; lower panel, 10 pg template/reaction) were obtained from Clontech Inc. Specific bands (303 bp) were PCR-amplified using intermedin gene-specific primer pairs under high-stringency conditions. The primer sequences for intermedin PCR analysis are: forward 5’- AGGGAGGGGAACCTCAGTTCAGGAG-3’ and reverse 5’- GTTCTTGTTCTTGCTGTCACTTGGGCCT-3’. The expression of GAPDH transcripts in different cDNA templates was also analyzed to assess the quality of the cDNA templates (higher panel, 1 ng template/reaction; lower panel, 10 pg template/reaction). Immunohistochemical staining of mouse stomach sections showed that intermedin is found primarily in the muscularis mucosae layer of stomach (B) and the signal is abolished by presaturation with the intermedin antigen (C). Specific signals are indicated by arrows. MU, mucosal layer; MS, muscularis layer; SL, serosal layer.

**Fig. 6. Decrease of systemic blood pressure and increase of heart rate by intermedin and related peptides.** A. Intermedin-long (IMDL), intermedin-short (IMDS), and ADM dose-dependently suppressed systolic blood pressure in male Sprague-Dawley rats. Blood pressure
change was monitored for 40 min, and averages at 10, 20, and 30 min were presented. **B.**

Increase in heart rate from treatment with different doses of IMDL, IMDS, or ADM in male Sprague-Dawley rats. **C.** Blockage of the hypotensive effect of intermedin by the CGRP receptor antagonist, CGRP8-37, and the putative intermedin receptor-binding domain peptide, IMD17-47. **D.** Suppression of blood pressure in male spontaneously hypertensive rats (SHR) by intermedin and the blockage of intermedin effects by receptor antagonists.

**Fig. 7. Suppression of food intake by intermedin in fasted mice.** Cumulative food intake in mice treated with saline (PBS, N=29), intermedin-long (IMDL, 100 nM/Kg, N=17), intermedin-short (IMDS, 100 nM/Kg, N=16), ADM (100 nM/Kg, N=20), or a type II CRH receptor-selective agonist SRP/urocortin II (15,23)(SRP, 100 nM/Kg, N=10) at 1, 2, and 4 h after treatment. *, significantly different from control animals injected with saline alone ($P < 0.05$).

**Fig. 8. Suppression of gastric emptying activity by intermedin.** Reduction of gastric emptying by intermedin-long (IMDL, 100 nM/Kg, N=24), intermedin-short (IMDS, 100 nM/Kg, N=20), ADM (100 nM/Kg, N=27), and SRP/urocortin II (100 nM/Kg, N=10) at 90 min after hormone treatment as compared to control animals receiving saline injection (N=31). Gastric emptying was calculated by comparing the stomach weight of treated mice to the stomach weight of control mice receiving no hormone treatment and killed at the time of hormone injection. Additional animals injected with saline and sacrificed at the same time as hormone-treated animals were used as experimental controls. *, significantly different from control animals injected with saline alone ($P < 0.05$).
Fig. 1C
Fig. 2A
Peptide (Log M)
cAMP production (pmol/ml)

- CGRP
- IMDS
- ADM
- IMDL

Fig. 2B
Fig. 2C

- Control
- 100 nM CGRP8-37
- 300 nM CGRP8-37

CAMP production (pmol/ml)
Fig. 2D

The graph shows the cAMP production (pmol/ml) in response to various concentrations of IMDL (nM) in the presence of IMD17-47, anti-IMD Ab, and anti-SRP Ab. The cAMP production is compared at 0, 10, and 30 nM IMDL, with significant increases indicated by asterisks (*) for IMD17-47 and anti-SRP Ab treatments.

- IMD17-47: * indicates a significant increase at 30 nM.
- Anti-IMD Ab: No significant increase is observed.
- Anti-SRP Ab: * indicates a significant increase at 30 nM.
Fig. 2E

125I-CGRP bound %

Cold ligand (x-fold)

ADM
CGRP
IMDL
IMDS

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Fig. 2F
Fig. 3

- A: CRLR
  - Calcitonin
  - ADM, CGRP, IMDS

- B: CRLR+RAMP1
  - ADM, CGRP, IMDS

- C: CRLR+RAMP2
  - ADM, CGRP, IMDS

- D: CRLR+RAMP3
  - ADM, CGRP, IMDS

Peptide (LogM) vs. cAMP production (pmol/ml)
Fig. 3
Fig. 4
Fig. 4
Fig. 4
Fig. 5
Changes in systolic blood pressure (mmHg)

Minutes

Fig. 6A
Change in heart rate (beats/min)

Minutes

Fig. 6B
Changes in systolic blood pressure (mmHg)

Fig. 6C
Changes in systolic blood pressure (mmHg)

Minutes

0 10 20 30

-50 -40 -30 -20 -10 0 10 20

IMDL 10 nM

ADM22-52 1 µM

IMDL 50 nM

IMDL 50 nM + ADM22-52 1 µM

CGRP8-37 1 µM

IMDL 10 nM + CGRP8-37 1 µM

IMDL 50 nM + CGRP8-37 1 µM

IMDL 50 nM

Fig. 6D
Fig. 7

Cumulative food intake (gm)

Time (Hours)

Saline
ADM
IMDS
IMDL
SRP
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