

Themed Section: Secretin Family (Class B) G Protein-Coupled Receptors –
from Molecular to Clinical Perspectives

REVIEW

Consequences of splice variation on Secretin family G protein-coupled receptor function

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Keywords

GPCR; Secretin family; family B; alternative splicing; receptor variant

Received

10 April 2011

Revised

13 June 2011

Accepted

14 June 2011

The Secretin family of GPCRs are endocrine peptide hormone receptors that share a common genomic organization and are the subject of a wide variety of alternative splicing. All GPCRs contain a central seven transmembrane domain responsible for transducing signals from the outside of the cell as well as extracellular amino and intracellular carboxyl termini. Members of the Secretin receptor family have a relatively large N-terminus and a variety of lines of evidence support a common mode of ligand binding and a common ligand binding fold. These receptors are best characterized as coupling to intracellular signalling pathways via $G_{\alpha s}$ and $G_{\alpha q}$ but are also reported to couple to a multitude of other signalling pathways. The intracellular loops are implicated in regulating the interaction between the receptor and heterotrimeric G protein complexes. Alternative splicing of exons encoding both the extracellular N-terminal domain as well as the extracellular loops of some family members has been reported and as expected these splice variants display altered ligand affinity as well as differential activation by endogenous ligands. Various forms of alternative splicing have also been reported to alter intracellular loops 1 and 3 as well as the C-terminus and as one might expect these display differences in signalling bias towards downstream effectors. These diverse pharmacologies require that the physiological role of these splice variants be addressed but should provide unique opportunities for drug design and development.

LINKED ARTICLES

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Abbreviations

AS, alternative splicing/alternative splice; CGRP, calcitonin gene related peptide (and receptor); CLR, calcitonin-like receptor; CRF₁, corticotropin releasing factor receptor 1; CRF₂, corticotropin releasing factor receptor 2; CT, calcitonin receptor or calcitonin; ECL, extracellular loop; EST, expressed sequence tag; GIP, gastric inhibitory polypeptide receptor or glucose-dependent insulinotropic polypeptide receptor; GLP-1, glucagon-like peptide receptor 1; GLP-2, glucagon-like peptide receptor 2; ICL, intracellular loop; PAC₁, pituitary adenylate cyclase activating peptide receptor; PACAP, pituitary adenylate cyclase activating peptide; PTH1, parathyroid hormone receptor 1; PTH2, parathyroid hormone receptor 2; RAMP, receptor activity modifying protein; UTR, untranslated region; VPAC₁, vasoactive intestinal peptide receptor 1; VPAC₂, vasoactive intestinal peptide receptor 2

Introduction

GPCRs are the largest gene family in eukaryotes comprising between 2% and 4% of the genome. GPCRs are responsible for transducing a wide range of extracellular stimuli including light, odours, hormones and neurotransmitters. Although the primary amino acid sequence homology is extremely low across GPCRs, they all share the same topology of an

extracellular N-terminus, a seven transmembrane (7TM) helix bundle and an intracellular C-terminus.

A subset of GPCRs is the family B, or Secretin family. In humans, these are a group of 15 peptide hormone receptors that form a distinct clade based on primary amino acid sequence (Fredriksson *et al.*, 2003). In some nomenclatures family B GPCRs include the 33 receptors (in humans) that are related to the Adhesion receptor so for clarity we will avoid

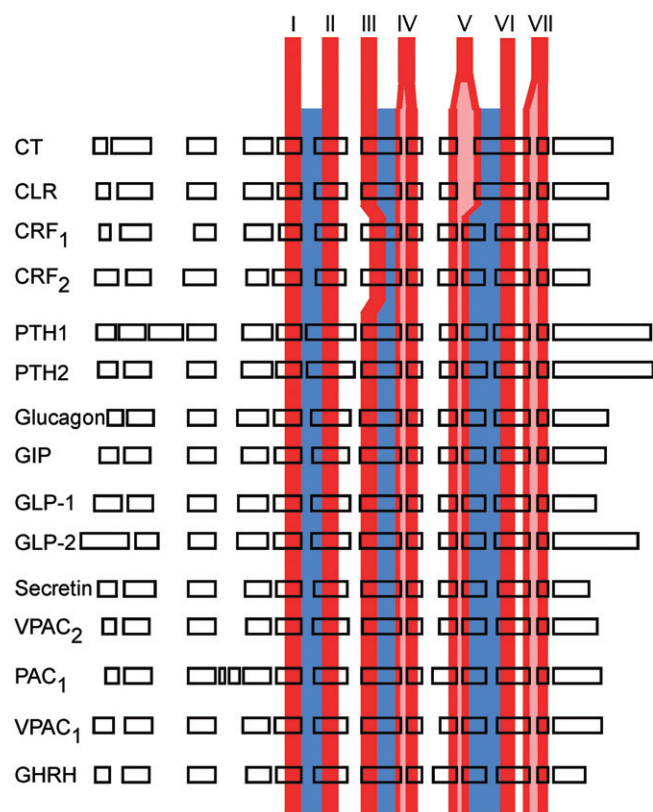


Figure 1

Schematic of the genomic organization of Secretin family GPCRs. Boxes indicate coding exons and are scaled according to amino acid length coded by each exon. The relative location of exons with respect to the overall receptor fold is depicted. The location of transmembrane helices is indicated in red (exon spanning helices shown with pink insert) and ICLs in blue.

using the term family B. There are two more bases for placing these receptors in a distinct family. First, they share a common genomic organization with four to six exons encoding the N-terminal domain and eight to nine exons encoding the 7TM bundle and C-terminus (see Figure 1), an arrangement that differs markedly from the genomic arrangement found in the related Adhesion, Glutamate and Frizzled receptor families. Second, the N-terminal domain of these receptors is very likely to form a common fold. The sequence homology in the N-terminus across this family is very low (Figure 2); however, the solution and crystal structures for isolated N-termini of corticotropin releasing factor receptor 2 β (CRF₂ β) (Grace *et al.*, 2004), pituitary adenylate cyclase activating peptide receptor (PAC₁) (Sun *et al.*, 2007), glucose-dependent insulintropic polypeptide receptor (GIP) (Parthier *et al.*, 2007), glucagon-like peptide receptor 1 (GLP-1) (Runge *et al.*, 2008), corticotropin releasing factor receptor 1 (CRF₁) (Pioszak *et al.*, 2008), parathyroid hormone receptor 1 (PTH₁) (Pioszak and Xu, 2008), CRF₂ α (Pal *et al.*, 2010) and calcitonin gene related peptide receptor [CGRP, a dimer between calcitonin-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1) (Haar *et al.*, 2010)] demonstrate a high conservation of secondary structure and a common mode of ligand binding (discussed below).

The Secretin family of GPCRs all respond to paracrine or endocrine peptide hormones that are typically in the range of 30–40 amino acids. These receptors are targets for existing drugs that treat osteoporosis [PTH and calcitonin receptor (CT)], hypercalcaemia (CT), Paget's disease (CT), type II diabetes (GLP-1, glucagon receptor) and are being actively pursued as targets for migraine (CGRP), depression and anxiety (CRF₁) and pancreatic diagnostics (Secretin receptor). Many of these receptors have multiple endogenous ligands and at least for the GLP-1, signalling bias of these endogenous ligands has been demonstrated (Koole *et al.*, 2010). Activation of these receptors regulates a wide variety of cellular physiology including cell cycle, differentiation, proliferation and release of other endocrine hormones. Their activation is most closely coupled to adenylate cyclase via G_{as} and to a lesser extent to PLC and intracellular calcium mobilization via G_{aq}, although their signalling is not confined to these pathways.

As has been mentioned above, the structure of the N-terminal extracellular domain of these receptors almost certainly forms a common fold. This common fold contains a small number of conserved residues (Figure 2) being aspartic acid, tryptophan, proline, glycine and tryptophan as well as six conserved cysteine residues, which form three conserved disulphide bridges (1–4, 2–5 and 3–6) (Grauschopf *et al.*, 2000; Perrin *et al.*, 2001; 2003; Bazarsuren *et al.*, 2002; Grace *et al.*, 2004; 2007; Lisenbee *et al.*, 2005; Parthier *et al.*, 2007; Sun *et al.*, 2007; Pioszak and Xu, 2008; Pioszak *et al.*, 2008; Runge *et al.*, 2008; Haar *et al.*, 2010). This structural information supports not only a common fold but also a common mode of binding, in which the carboxy end of the ligand interacts with the N-terminal domain of the receptor. Experiments using chimeric receptors and ligands for CT/glucagon receptor (Stroop *et al.*, 1995), Secretin/vasoactive intestinal peptide receptor 1 (VPAC₁) (Holtmann *et al.*, 1995), CT/PTH₁ (Bergwitz *et al.*, 1996), GIP/GLP-1 (Gelling *et al.*, 1997), glucagon/GLP-1 (Runge *et al.*, 2003) support a model in which the amino end of the ligand interacts with the juxtamembrane region and extracellular loops (ECL) and are consistent with the structural data. Progressive truncation of the N-terminus of Secretin receptor family ligands progressively converts ligands for VIP (Turner *et al.*, 1986), PTH₁ (Goldman *et al.*, 1988), CGRP (Wang *et al.*, 1991), CT (Feyen *et al.*, 1992), GIP (Tseng *et al.*, 1996) and GLP-1 (Montrose-Rafizadeh *et al.*, 1997) from agonists into antagonists demonstrating that although, in many cases, the C-terminus is required for high affinity binding, it is the N-terminus that is competent to stabilize the active conformation of these receptors. Secretin family ligands therefore interact in a bivalent mode with the receptor, the N-terminal receptor domain providing a high affinity site for the peptide carboxy terminus that constrains the lower affinity interaction of the amino terminus of the peptide with the juxtamembrane domain and ECLs to stabilize the active receptor conformation. The active conformation of the receptor then acts as a guanine nucleotide exchange factor for the G_α subunit of the trimeric G protein complex. There is no direct evidence that addresses the regions of the cytoplasmic face of GPCRs that are responsible for interactions with the trimeric G protein complex. A large amount of mutational data as well as inferences made from crystal structures of both family A

[illegible]

Figure 2

Alignment of the extracellular N-termini of Secretin family GPCRs. Amino acids encoded by alternative exons are shown in alternating black and blue text with amino acids encoded by two exons shown in red text. Predicted signal peptides are highlighted as italicized, underlined text. The six conserved cysteine residues are highlighted by red background and have the cysteine alpha helices are indicated with H, beta strands with B and beta turns with T. The disulphide bonding pattern for all receptors is 1-4, 2-5 and 3-6. The other conserved residues are highlighted in purple. Above the alignment

GPCRs and G proteins predict that intracellular loops (ICL) 2 and 3 as well as the C-terminal tail are involved in this interaction (reviewed in Huang and Tesmer, 2011).

Alternative splicing in Secretin family receptors

The genomic organization exhibited by the Secretin family of GPCRs engenders the potential for a wide variety of alternative splicing (AS). As it transpires, almost all combinations of AS that one might imagine appear to have evolved within this family (Table 1 and Figure 3).

The current nomenclature of AS within the Secretin family has a number of inconsistencies that make it difficult to follow. For example, the CT receptor has splice variants CTa and CTb, which in human refer to AS of the first ICL but in rat refer to AS in the first ECL. The PAC1 receptor has a wide variety of alternatively spliced variants with notations such as normal, short, very short, hip, hop and hiphop, which can be difficult for those unfamiliar with the literature to follow. We would recommend the adoption of a standardized nomenclature such as that suggested by Sammeth (Sammeth *et al.*, 2008), although this has issues with the complexity of the nomenclature and its routine use.

In addition to AS of the Secretin family it should be noted that proteins directly interacting with this family, including RAMPs, G α , G β , GPCR kinases and arrestins, are also subjected to AS of their coding regions potentially adding greatly to the possible diversity of receptor pharmacologies.

AS in the 5' untranslated region

The 5' untranslated region (UTR) of mRNA species is able to regulate their stability as well as the efficiency of translation initiation. Changes in the 5' UTR can result in alternative coding regions and these will be discussed in the section on AS of the N-terminus.

The human Ensembl/Havana merge database of validated transcripts includes mRNA with alternative non-coding 5' UTRs for CT, CLR, CRF₂, PTH1 and PAC1. The alternative 5' UTR of CT is the result of the use of an alternative osteoclast specific promoter and there is no evidence whether this alternative 5' UTR confers any difference in stability or translational efficiency. The nature and function of the AS 5' UTR in other human Secretin family receptors has yet to be determined.

AS in the N-terminus

In a bivalent ligand binding model, the receptor N-terminus serves as a high affinity bait for the carboxy terminus of the ligand. This then brings the amino terminus of the ligand in close proximity with the ECLs of the receptor where it is able to stabilize the receptor active state. In this model AS of the N-terminus of the receptor would enable new receptor variants to be generated. These could recognize alternative

ligands that differed in their carboxy but not amino terminal end, alter the rank order of affinities a particular receptor had for multiple endogenous ligands or even generate alternative signalling bias from multiple endogenous ligands. Alternative N-termini have been reported for CRF₁, CRF₂, PAC₁ and the Secretin receptor.

Splice variants in which the third coding exon is skipped ($\Delta 3e$) have been reported for CRF₁ [CRF_{1c} (Ross *et al.*, 1994)], PAC₁ [PAC_{1vs} (Dautzenberg *et al.*, 1999)] and Secretin (Figure 3 and Table 1) (Ding *et al.*, 2002b). The third coding exon contains the 2nd, 3rd and 4th conserved cysteines and its deletion may lead to incorrect folding (see Figure 2). Consistent with a poorly functional N-terminus, CRF has very low potency at CRF_{1c} compared with CRF α and CRF_{1c} did not bind CRF at concentrations tested (Ross *et al.*, 1994), this is in spite of the fact that CRF_{1c} does appear to traffic correctly to the cell surface (Zmijewski and Slominski, 2009b). The function of CRF_{1c} has not been established although its expression is regulated by cell density (Zmijewski and Slominski, 2009a). CRF₁ has been reported to form proximers¹ (Kraetke *et al.*, 2005) and also displays biphasic responses attributable to coupling to two different G protein pools (Wietfeld *et al.*, 2004). This being the case, co-expression of CRF_{1c} with CRF α could alter the signalling profile, but this remains untested. In the case of the Secretin receptor, endogenous co-expression of full-length and $\Delta 3e$ mRNA in pancreatic carcinoma cell lines results in at least a three order of magnitude reduction in Secretin potency (Ding *et al.*, 2002a). This can be recovered by transfection of increasing amounts of wild-type receptor (Ding *et al.*, 2002a). This pseudo dominant negative effect of the $\Delta 3e$ Secretin receptor is very difficult to explain; Ding *et al.* (2002a) provide BRET data showing the wild-type and $\Delta 3e$ receptors form a proximer alluding that interaction between the two receptor forms causes the observed loss of affinity (Ding *et al.*, 2002b) and potency (Ding *et al.*, 2002a). Elsewhere homo-dimerization of the Secretin receptor has been validated through combined disruption of BRET signal through mutation of the dimerization interface, disruption of BRET signal through the use of peptides that mimic the dimerization interface and cysteine disulphide bonding across TM regions of the receptor (Hari-kumar *et al.*, 2007; 2008; Gao *et al.*, 2009). Nonetheless, in the absence of direct data on the relative expression of the mature proteins at the cell surface as well as composition and stoichiometry of receptor proximers, interpretation of the severe loss of binding/signalling is extremely difficult. If we accept that endogenously all proximers contain both receptor variants, the implication is that the N-terminus of wild-type receptor provides strong cooperativity for ligand binding. Currently, the level of cooperativity reported across the Secretin dimer (Gao *et al.*, 2009) appears insufficient to explain the magnitude of affinity and potency loss engendered by co-expression of the $\Delta 3e$ variant. The PAC₁ receptor is

¹We have chosen to use the term proximer to denote the higher order complex whose existence is demonstrated by resonance energy transfer methods such as BRET and FRET. The distance over which energy resonance transfer operates is 1–10 nm. For comparison the height of the lipid bilayer is approximately 4 nm and the width of a GPCR is about 3.8 nm, thus resonance energy transfer could conceivably occur between two GPCRs separated by two to three intervening GPCRs or similarly sized proteins.

Table 1

Summary of splicing variants of Secretin family GPCRs that affect the coding region

Receptor	Variation	Effect	Comment
N-termini			
CRF ₁ c	Coding exon 3 skipped	Severe reduction in ligand affinity	Supported
CRF _{2α}	Alternative exon encoding 1st N-terminal alpha helix	Functional	Supported
CRF _{2β}	Alternative exon encoding 1st N-terminal alpha helix	Functional	Supported
CRF _{2γ}	Alternative exon encoding 1st N-terminal alpha helix	Functional	Supported
PAC ₁ 3a	Exon insertion in N-terminal loop 1, between the first alpha helix and beta strand	Functional	Supported in rat, unsupported in human
Secretin receptor			
CTRΔ47N	Coding exon 3 skipped	Severe reduction in ligand affinity, dominant negative	Supported
	Apparent cryptic splice donor from 5' UTR, skip coding exon 1	No reduction of potency for calcitonins but reduced potency for amylin suggesting interaction with RAMP lost	Unsupported
Headless			
CRF ₁ e2	Alternative first exon use of coding exon 5, receptor starts at TM1	None reported	Unsupported
CRF ₁ h2	Cryptic first exon followed by coding exon 5 creating N-terminally truncated receptor	None reported	Unsupported
CTRheadless	Alternative first exon results in N-terminally truncated receptor	Severe reduction in ligand affinity, dominant negative	Supported in teleost fish but not orthologous to mammalian CT receptor genes
ECLs			
CT receptor	Insertion of extra exon in ECL1	Reduced affinity	Supported in rat, unsupported in human
ICLs			
CT receptor	Insertion of extra exon in ICL1	Signalling bias, reduced internalization	Supported
CTR7a	Cryptic additional exon 7 resulting in frame shift and stop codon. Predicted to generate N-terminus plus TM1	None reported	Unsupported
CRF ₁ β	Insertion of extra exon in ICL1	Reduced affinity and potency, increased internalization	Supported
PAC ₁ hip	Insertion of extra exon in ICL3	Signalling bias	Supported
PAC ₁ hop ₁	Insertion of extra exon in ICL3	Signalling bias	Supported
PAC ₁ hop ₂	Insertion of extra exon in ICL3	Signalling bias	Supported in rat, proposed but unsupported in human
PAC ₁ hiphop ₁	Insertion of two extra exons in ICL3	Signalling bias	Supported
Tailless			
CRF ₁ var	Coding exon 14 skipped, loss of half of TM7 then frame shift to stop codon equivalent to δ13e of CTR	No effect on affinity, impaired signalling	Unsupported
PTH1	Coding exon 14 skipped, loss of half of TM7 then frame shift to stop codon equivalent to δ13e of CTR	Affinity not reported, impaired trafficking, insufficient signalling data to ascribe function	Unsupported
CTR δ13e	Coding exon 13 skipped, loss of half of TM7 then frame shift to stop codon	No effect on affinity, cAMP stimulation unaffected impaired signalling to IP ₃	Reported in rabbit, unsupported in human

Those splice variants listed as supported have supporting evidence from more than one group and/or organism as well as supporting EST data in the Ensembl/Havana merge of validated transcripts and/or EcGene.

Those splice variants listed as unsupported do not have supporting EST data in the Ensembl/Havana merge of validated transcripts and/or EcGene.

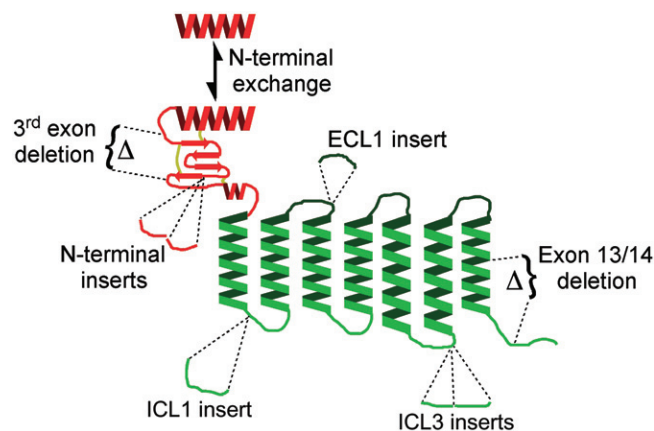


Figure 3

Cartoon depicting overall topology of Secretin family GPCRs. The N-terminal domain is shown in red with the seven transmembrane bundle in green. The relative locations of AS reported for the family are shown. N-terminal domain exchange is a feature of CRF₂α, CRF₂β and CRF₂γ. CRF₁, PAC₁ and Secretin receptors have 3rd exon deletion variants. PAC₁ has both N-terminal and ICL3 insert variants. CT receptor has variants with ICL1 insert, ECL1 insert and exon 13 deletion and CRF₁ has an ICL1 insert variant.

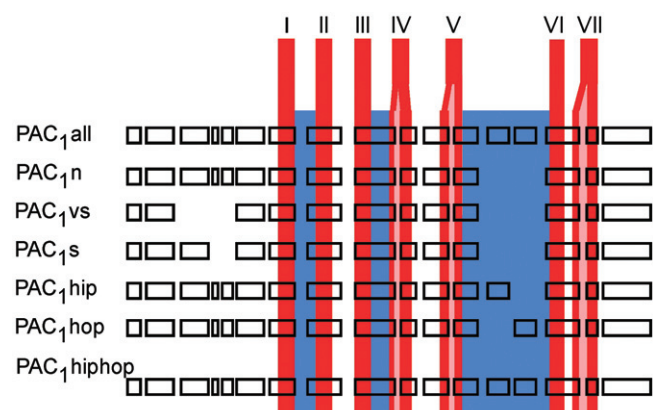


Figure 4

Schematic of the genomic organization of PAC₁ receptor indicating the human splice variants discussed in this review. The top most exon pattern labelled all shows all coding exons for human PAC₁; however, we are not aware of a splice variant that incorporates all these exons. Boxes indicate coding exons and are scaled according to amino acid length coded by each exon. The relative location of exons with respect to the overall receptor fold is depicted. The location of transmembrane helices is indicated in red (exon spanning helices shown with pink insert) and ICLs in blue.

unusual in this family as the variant usually referred to as 'normal' contains six coding exons for the N-terminal domain (Figures 2 and 4). This is the result of the inclusion of two small exons between those corresponding to the 3rd and 4th of other family members (Figure 2 and PAC_{1n} Figure 4). PAC_{1vs} has exon 3 as well as the extra exons, with no equivalent in other family members, deleted (Figure 4) and is thus equivalent CRF_{1c} and Δ3e Secretin receptors above. This

variant displays a two order of magnitude reduction in affinity for its ligands pituitary adenylate cyclase activating peptide (PACAP) 38 (comprising 38 amino acids) and 27 (the same peptide with a C-terminal truncation) relative to the normal receptor variant (Dautzenberg *et al.*, 1999), as well as a 20- to 100-fold decrease in potency for cAMP production (Dautzenberg *et al.*, 1999; Lutz *et al.*, 2006). The mRNA encoding this variant is co-expressed with full-length and short (see later, exon 3 present, unique exons absent) PAC₁ in neuroblastoma cell lines SH-SY-5Y and Kelly cells (Lutz *et al.*, 2006). The potency of PACAP38 to elicit a cAMP response in these cell lines is consistent with the potencies observed in heterologous systems for PAC_{1n} and PAC_{1s} (Lutz *et al.*, 2006), suggesting that PAC_{1vs} does not exert a dominant effect in this pathway.

In the above cases of CRF_{1c}, Δ3e Secretin receptor and PAC_{1vs} it is not clear what physiological relevance would be engendered by their pharmacology. In each case the N-terminus would be predicted to fold incorrectly and the effect on wild-type receptor signalling (if any) could be achieved simply by altering wild-type receptor expression. In comparison, the AS that gives rise to CRF₂α, β and γ and PAC_{1n} and PAC_{1s} variants results in alternative N-termini that are all fully functional and elicit altered pharmacology. For CRF₁, AS arises through the use of different promoters that drive expression of alternative first exons, with CRF₂α containing a single first exon that is 5' proximal to the first common exon and CRF₂β containing two further upstream exons before splicing to the common first exon, CRF₂γ contains a 3rd alternative first exon located between 5' α β exons and again splices to the same common exon. The consequence of this AS is that the N-terminal sequence containing the first conserved cysteine differs between the three forms with protein lengths of 411, 438 and 397 amino acids respectively (Figure 3, N-terminal exchange). The crystal structure of CRF₂α shows an alpha helix extending either side of the first conserved cysteine before the loop that connects it with the first beta strand (Pal *et al.*, 2010). This contrasts with the NMR solution structure of CRF₂β in which the corresponding structural element forms a disordered loop that is constrained by the disulphide bond and its link with the first beta strand (Grace *et al.*, 2007). The N-terminus of CRF₂γ has not been solved; however, the existing structural studies demonstrate that the interaction between the ligand and N-terminal domain occurs via the part of this fold opposite the AS sequence. In this light, the observation that these CRF₂ variants have identical binding properties is unsurprising (Kostich *et al.*, 1998; Ardati *et al.*, 1999). These two studies have examined different downstream outputs from receptor activation. Kostich *et al.* (1998) measured cAMP accumulation and reported pEC₅₀ values for CRF, sauvagine, urotensin and urocortin, which did not differ between CRF₂α and γ but showed 10-fold higher potency at CRF₂β. This is in contrast to the report of Ardati *et al.* (1999), who used a cAMP response element driven reporter assay and showed identical potencies of CRF₂α and β with respect to CRF, sauvagine, urotensin and urocortin with pEC₅₀ values consistent with the higher potency values established for CRF₂β by Kostich *et al.* (1998). This difference may be due to differences in receptor reserve in the two assays. The β and γ variants show a more restricted pattern of tissue expression compared with the α variant;

however, with the existing molecular pharmacology, it is unclear why the different isoforms exist. One possibility may be that CRF₂ can form a complex with one or more RAMP isoforms. The crystal structure of the N-terminal domains of CLR in complex with RAMP1 shows extensive contacts between RAMP1 and the alpha helix of CLR (Haar *et al.*, 2010), which corresponds to the AS region of the CRF₂ α , β and γ variants. AS splicing could, therefore, regulate the interaction between CRF₂ and RAMPs thereby regulating ligand selectivity. As mentioned above, PAC₁ is unusual among Secretin family members in that there is a common receptor variant (PAC_{1n}) for which the N-terminus is encoded by six exons (Figure 3, C-terminal of the two N-terminal inserts and Figure 4). AS of this region to remove coding exons 4 and 5 results in a receptor, PAC_{1s} (Figure 4), with exon organization that mimics the remainder of the family. PAC₁, VPAC₁ and VPAC₂ all respond to physiologically relevant concentrations of PACAP; however, PAC₁ is normally considered a type I PACAP receptor due to the low affinity and potency that VIP (vasoactive intestinal peptide) displays at this receptor. The first molecular pharmacological description comparing PAC_{1n} and PAC_{1s} was performed in HEK293 cells and indicated that VIP displays low affinity and potency for cAMP production only at the PAC_{1n} variant and PAC_{1s} does not show the same ligand selectivity (Dautzenberg *et al.*, 1999). In contrast to these results a follow-up study performed in CHO cells showed that VIP had low affinity and potency for cAMP production at both PAC_{1n} and PAC_{1s} (Ushiyama *et al.*, 2007). This, combined with the observation that RAMP2 selectively interacts with VPAC₁ and alters its pharmacology (Christopoulos *et al.*, 2003), suggests that AS of the N-terminal domain of PAC₁ could yield receptors with significantly altered pharmacology by regulating the interaction with RAMP proteins. In rat testis an additional functional N-terminally spliced variant of PAC₁ has been reported, PAC₁3a (Daniel *et al.*, 2001). This variant has, in addition to the six exons that encode the N-terminus of PAC_{1n}, an additional exon between coding exons 3 and 4 adding another 24 amino acids to the N-terminal domain (Figure 3, N-terminal of the two N-terminal inserts). At PAC₁3a PACAP27 displays equivalent affinity but slightly reduced potency for cAMP and IP₃ production when compared with PAC_{1n} (Daniel *et al.*, 2001). PACAP38 displays higher affinity but significantly lower efficacy at PAC₁3a compared with PAC_{1n} (Daniel *et al.*, 2001) suggesting the higher affinity has been achieved in part by stronger G protein coupling. This variant has not been reported in humans.

Headless receptors

The expression of an N-terminally deleted, or headless, Secretin family GPCR in isolation would result in a non-functional receptor. If, on the other hand, a headless receptor was expressed that was competent to form homodimers with a full-length counterpart or heterodimers with other family members then altered pharmacology could result through, for example, loss of cooperativity.

In humans there are no examples of a headless Secretin family GPCR, nor is there evidence for such receptors through expressed sequence tag (EST) clustering in either

Ensembl/Havana merge or ECGene. In spite of this, the existence of headless CRF₁ variants has been proposed (Zmijewski and Slominski, 2010). The CT receptor, which has been shown to form a proximer (Harikumar *et al.*, 2010), has been reported to have an AS variant in pufferfish that lacks the N-terminal domain (Nag *et al.*, 2007a). These authors subsequently reported that this N-terminally truncated variant is able to act in a dominant negative manner (Nag *et al.*, 2007b). These reports are unlikely to have any relevance to any mammalian Secretin family GPCR. Teleost fish are well documented to have undergone at least one whole-genome duplication subsequent to their divergence from the mammalian lineage (e.g. Jaillon *et al.*, 2004). There appear to be four CT receptor genes in the teleost lineage (documented for zebrafish, medaka, pufferfish and stickleback) with two sharing similar genomic organization and total protein length when compared with the mammalian CT receptor. The CT receptor orthologue studied by this group has 23 exons and a predicted length of 794 amino acids and should not be considered as orthologous to human.

Although an attractive means to regulate receptor function we believe there is no evidence to support the existence of N-terminally truncated human Secretin family GPCRs.

Soluble N-termini

As has been discussed, the N-terminal domain is capable, in some cases, of high affinity ligand binding in its own right. Expression and secretion of soluble N-termini would therefore be predicted to sequester ligand and reduce its local effective concentration. If soluble N-terminal domains were stored in secretory vesicles and released in response to stimuli, including their own ligand, this would provide a means to provide additional temporal and spatial control over receptor signalling.

In humans the existence of an mRNA that may code for a soluble N-terminus of CRF₁ has been reported (Pisarchik and Slominski, 2001); however, there is no supporting evidence for this variant in either Ensembl/Havana merge or ECGene. AS mRNA species predicted to code for soluble N-termini of both CRF₁ and CRF₂ have been reported in mouse (Pisarchik and Slominski, 2001; Chen *et al.*, 2005). The data related to these forms are consistent with the possibility that they would be secreted and their action would be to reduce the effective concentration of available ligand (Perrin *et al.*, 2001; Pisarchik and Slominski, 2001; Chen *et al.*, 2005; Evans and Seasholtz, 2009; Zmijewski and Slominski, 2009a,b). At present we would regard the data related to these soluble N-termini as purely phenomenological.

AS of ECLs

The current model for Secretin family ligand–receptor interaction predicts the ECLs to be important in stabilizing the active state of the receptor–ligand complex. AS of these loops would therefore provide another means to alter pharmacology through altered ligand selectivity or altered signal transduction. It is evident from the genomic structure that

insertions could be accommodated in all ECLs, with the exception of CT receptors and CL receptors that have the equivalent of coding exons 10 and 11 fused. Currently there is no evidence for such AS in humans through EST clustering in either Ensembl/Havana merge or ECGene.

In the rat brain, alternatively spliced variants of the CT receptor have been identified in which an extra exon is inserted between the 6th and 7th coding exon to create a receptor variant containing an additional 37 amino acids in ECL1 (ECL1+) (Figure 3) (Albrandt *et al.*, 1993; Sexton *et al.*, 1993). This variant displayed 50-fold lower affinity for the non-endogenous ligand salmon CT whereas rat CT showed no binding at the concentrations tested (Houssami *et al.*, 1994). In a HEK293 background ligands displayed cAMP accumulation potencies consistent with binding affinities at both wild-type and ECL1+ receptors (Houssami *et al.*, 1994). Contrastingly, in a *Xenopus* oocyte background, in which cystic fibrosis transmembrane conductance regulator transactivation was used as a measure of cAMP production, both receptor variants displayed equivalent responses to salmon CT but not human CT (Matsumoto *et al.*, 1998). This variant has not been tested for interaction with RAMP proteins nor for its ability to act as a receptor for amylin.

AS of ICLs

The intracellular face of GPCRs provides the interaction surface for a wide range of proteins involved in signalling and regulation. Predictions based on mutagenesis studies would suggest that ICL2, ICL3 and the C-terminus would be involved in interactions with G proteins, while ICL1 may serve a regulatory role. Splice variants that alter ICLs could then provide a means of regulating receptor function. Additionally, GPCRs can be thought of as transducers of signals across the plasma membrane such that alterations on the intracellular face could result in differences in ligand affinity.

Alternative splicing of ICL1 has been reported for both CT and CRF₁ receptors (Figure 3). In both cases the AS spliced version results from inclusion of an extra exon that encodes 16 [CT (Gorn *et al.*, 1992)] or 29 [CRF₁ (Chen *et al.*, 1993)] additional amino acids. The CT ICL1+ receptor displayed no difference when compared with ICL1- in affinity for its ligands (Moore *et al.*, 1995; Nussenzveig *et al.*, 1995) but displayed impaired coupling to G_{as} as assessed by an approximate 100-fold decrease in potency of CT to stimulate cAMP production (Moore *et al.*, 1995; Nussenzveig *et al.*, 1995). The ICL1+ variant of the CT receptor was not able to couple to intracellular calcium release (presumably via G_{aq}) and displayed impaired internalization when compared with the ICL1- variant (Moore *et al.*, 1995). This study was performed in the baby hamster kidney cell background. Experiments in a HEK293 background showed a loss of coupling to intracellular calcium release and cAMP accumulation for the ICL1+ CT receptor variant (Raggatt *et al.*, 2000). Although cell background-dependent variations in responses are observed, unpublished data from our laboratory confirmed impaired coupling of the CT receptor ICL1+ variant in stable transfectants of HEK293, mouse fibroblast (3T3) and African green monkey kidney (Cos7) cell lines suggesting the observed change in cAMP potency is an intrinsic property of this recep-

tor variant. In contrast studies on the CRF₁ ICL1+ are not as straightforward to interpret. The initial report comparing CRF₁ ICL1+/- in a transiently transfected Cos7 system reported a twofold reduction in affinity of CRF for the ICL1+ receptor and an approximate 100-fold reduction in potency (Xiong *et al.*, 1995). A subsequent study by the same group of stable transfectants of ICL+ and - variants in LLC-PK-1 cells (pig kidney) showed a fourfold to fivefold decrease in affinity and an approximate 10-fold decrease in both potency and efficacy of CRF for cAMP accumulation at the ICL1+ variant (Nabhan *et al.*, 1995). In radioligand binding on isolated membranes, GTPγS caused an apparent decrease in the affinity of CRF₁ ICL- but not ICL+ for CRF (Nabhan *et al.*, 1995), providing direct support for decreased G protein coupling of the ICL1+ variant. Two recent reports from a different group reported a fourfold to fivefold reduction in affinity of the CRF₁ ICL1+ variant [transient transfection CHO, transient and stable HEK293 (Markovic *et al.*, 2006; Teli *et al.*, 2008)] with similar receptor levels with no significant difference in potency of CRF for cAMP stimulation but rather a difference in efficacy (Markovic *et al.*, 2006; Teli *et al.*, 2008). These studies also conclude that the ICL1+ variant of CRF₁ is more sensitive to desensitization and internalization in a PKC-dependent fashion, in contrast to the increased resistance to internalization of the ICL+ CT receptor variant (Moore *et al.*, 1995). Both CT and CRF₁ ICL1+ variants display a more restricted tissue expression compared with their ICL1- counterparts; however, there is currently no data that demonstrate whether individual cells express only single or both receptor variants. Both CT and CRF₁ receptors have been shown to form higher order complexes but without data on AS expression in native tissues it is not possible to speculate as to the physiological role of the ICL1+ receptors. Clearly there is a need for experiments on native tissues.

PAC₁ is subject to AS of ICL3 (Figures 3 and 4). ICL3 inserts generated by AS of two additional exons were originally identified in rat (Spengler *et al.*, 1993), then subsequently in human (Pisegna and Wank, 1996). The 5' of these extra exons has been termed hip (Figure 4) and encodes an extra 28 amino acids (Spengler *et al.*, 1993; Pisegna and Wank, 1996). The 3' exon has been termed hop (Figure 4) and in humans also encodes an extra 28 amino acids (Pisegna and Wank, 1996), although in rat has been shown to encode 28 (hop1) or 27 (hop2) amino acids through the use of AS acceptor sites (Journot *et al.*, 1995). In rat and mouse PAC₁ ICL3 variants significant differences in pathway coupling are observed. No PAC₁ variants studied were able to alter IP₃ concentration when stimulated by PACAP27 whereas PACAP38 can stimulate IP₃ equally via PAC_{1n} and PAC_{1hop}, to a lesser extent PAC_{1hiphop} and not at all through PAC_{1hip} (Spengler *et al.*, 1993; Journot *et al.*, 1995; Ushiyama *et al.*, 2007). In contrast human PAC₁ ICL3 isoforms show little or no difference in their abilities to stimulate cAMP or IP₃ production in response to either PACAP38 or 27 (Pisegna and Wank, 1996; Pisegna *et al.*, 1996; Lutz *et al.*, 2006). The amino acid sequence encoded by rat and human hop1 exons is identical, whereas the sequence encoded by hip differs by two non-conservative substitutions of alanine (rat) to threonine and proline (rat) to leucine. The substantial signalling differences reported between rat and human PAC₁ ICL3 variants could therefore result simply from these substitutions or may

be a result of cell background effects as has been discussed above. In addition to the reports describing coupling of rat and human receptors via effectors to stimulate cAMP and IP₃ production, there have been two reports from the same group describing coupling of bovine PAC₁ to voltage gated calcium channels (VGCCs) (Mustafa *et al.*, 2007; 2010). In this case both PACAP38 and 27 show similar affinities and potencies for cAMP accumulation via PAC₁hop and PAC₁n; however, only PAC₁hop is able to couple to VGCCs (Mustafa *et al.*, 2007). In addition, it is coupling via VGCCs that is required for PACAP-stimulated catecholamine release, implicating PAC₁hop as the physiologically important isoform in acute adrenal stress response. These data have yet to be reported for human PAC₁ variants; however, the bovine hop cassette encodes the identical amino acid sequence to the human cassette.

Tailless receptors

The 7th transmembrane domain of Secretin family members is encoded by two exons. The second of these exons encodes the last 14 amino acids of this transmembrane domain (Figures 1 and 3). Skipping of this exon in humans has been proposed for CRF₁ (Markovic *et al.*, 2008) and PTH1 (Alonso *et al.*, 2011). For both receptors this is proposed to result in a 6TM receptor that displays impaired trafficking (Alonso *et al.*, 2011) with CRF₁ displaying similar ligand affinity to the full-length CRF variants but impaired signalling (Markovic *et al.*, 2008). An equivalent CT receptor has been reported in rabbits (termed δ 13e). This CT receptor variant displays similar ligand affinity to full-length CT with less than a twofold reduction in its ability to stimulate cAMP production in response to hCT but complete loss of ability to stimulate IP₃ accumulation. Currently insufficient data exist regarding this type of variant to make any conclusion about pharmacological or physiological relevance.

Discussion

A wide array of AS events have been reported for the Secretin family of GPCRs. These splice variants do, indeed, display a range of altered pharmacologies and there is little doubt that the variants identified in this family engender diverse alternative receptor phenotypes, which warrant careful examination. It is clear that AS is capable of generating new receptor types that are able to distinguish different endogenous ligands as well as couple to alternative intracellular second messenger pathways and/or alter receptor regulation. This is likely to be extremely important physiologically for different cell types to respond appropriately to the same endocrine ligand. Moreover, understanding the location and nature of signalling from alternatively spliced receptor variants provides the opportunity to design more effectively targeted pharmaceuticals.

In spite of this very positive view there are a number of limitations in almost all the literature published on the molecular pharmacology of these variants. The literature relating to expression of AS Secretin family receptors almost

exclusively examines expression at the tissue and mRNA level. Many of the studies cited, and splice variants reported are proposed to exert alternative pharmacology through their interaction with other splice variants of the same receptor. There are very few instances, however, in which co-expression of both variants has been convincingly demonstrated. There is also an underlying assumption that presence, or even quantitation, of various AS mRNA species corresponds with expression of mature protein at the cell surface. In this regard there is an urgent need for both high-quality pan splice variant specific antibodies as well as antibodies generated capable of distinguishing between splice variants. This is especially true in the case of examining transformed or primary cell lines that endogenously express more than one variant of a particular receptor. Presently the research in this field takes the form of identification of AS mRNA species followed by heterologous expression in model cell lines to examine the resulting molecular pharmacology. This is an entirely reasonable approach; however, a number of theoretical AS variants have been published for which little or no supporting evidence exists (these have been omitted from this review). Having established the pharmacology by the above method studies do need to be extended. Experiments in transformed cell lines that endogenously express receptor splice variants need to be performed to assess cell background-dependent changes in receptor activity. In addition experiments on primary cells harbouring endogenous receptors as well as endogenous combinations of splice variants are necessary if the molecular pharmacology is to be related to receptor physiology. Indeed, the physiological relevance of even the best characterized splice variants is unknown simply because there are effectively no data on which splice variants are endogenously expressed in particular cell types.

Acknowledgements

This work was funded by Program Grant 519461 and Project Grant 436780 from the National Health and Medical Research Council (NHMRC) of Australia. AC is a Senior, and PMS a Principal Research Fellow of the NHMRC.

Conflict of interest

None declared.

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