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Review

Emerging role of alternative splicing of CRF1 receptor in CRF signaling

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Alternative splicing of mRNA is one of the most important mechanisms responsible for an increase of the genomic capacity. Thus the majority of human proteins including G protein-coupled receptors (GPCRs) possess several isoforms as a result of mRNA splicing. The corticotropin-releasing factor (CRF) and its receptors are the most proximal elements of hypothalamic-pituitary-adrenal axis (HPA) — the central machinery of stress response. Moreover, expression of CRF and regulated activity of CRF receptor type 1 (CRF1) can also play an important role in regulation of local stress response in peripheral tissues including skin, gastrointestinal tract or reproductive system. In humans, expression of at least eight variants of CRF1 mRNA (α, β, c, d, e, f, g and h) was detected and alternative splicing was found to be regulated by diverse physiological and pathological factors including: growth conditions, onset of labor, during pregnancy or exposure to ultraviolet irradiation. The pattern of expression of CRF1 isoforms is cell type specific and recently has been linked to observed differences in responsiveness to CRF stimulation. In the proposed model of regulation of CRF-signaling, isoform CRF1α plays a central role. Other isoforms modulate its activity by oligomerization, leading to alteration in receptor trafficking, localization and function. Co-expression of CRF1 isoforms modulates sensitivity of cells to the ligands and influences downstream coupling to G-proteins. The other possible regulatory mechanisms include fast mRNA and/or protein turnover or decoy receptor function of CRF1 isoforms. Taken together, alternative splicing of CRF1 can represent another level of regulation of CRF-mediated stress responses at the central and peripheral levels. Chronic stress or malfunction of the HPA-axis have been linked to numerous human pathologies, suggesting that alternative splicing of CRF1 receptor could represent a promising target for drugs development.

Keywords: corticotropin-releasing factor, corticotropin-releasing factor receptor type 1, G-proteins, GPCR, hypothalamus-pituitary-adrenal axis, skin stress.

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HPA AXIS

Organisms do not always live under optimal conditions and are frequently subjected to various external and internal stimuli, thus during evolution a global system of a stress response was developed. In mammals, the hypothalamus-pituitary-adrenal axis (HPA) plays the central role in the regulation of stress response by production and secretion of important regulatory peptide intermediates and steroid hormones (Fig. 1). The classic signaling cascade of stress response is triggered by the synthesis of corticotropin-releasing factor (CRF, CRH) in the hypothalamus (Vale *et al.*, 1981; Smith & Funder, 1988; Chrousos & Gold, 1992). Activation of CRF receptor type 1 (CRF1) in the anterior pituitary results in production of pro-opiomelanocortin (POMC) with its subsequent processing to adrenocorticotropin (ACTH), and lipotropins (LPH) (Vale *et al.*, 1981; Smith & Funder, 1988). LPH can be further processed to β-endorphin, while ACTH to α-melanocyte-stimulating hormone (MSH). ACTH is responsible for stimulation of production and secretion of steroids in adrenal glands (cortisol–human, corticosterone–rodents) and this process is mediated by melanocortin receptor type 2 (MC2). MSH stimulates melanogenesis in the skin *via* interaction with MC1 receptor (MC1) (Pawelek *et al.*, 1992; Abdel-Malek *et al.*, 2000; Slominski *et al.*, 2004b; Bohm *et al.*, 2006). β-Endorphin is a powerful agonist of the μ-opioid receptor and similarly to other opioids works as an analgesic, promoting good mood and relaxation factors (Charmandari *et al.*, 2005). Circulating cortisol inhibits synthesis of CRF (Roche *et al.*, 1988) and POMC forming a negative feedback, responsible for maintaining of body homeostasis (for reviews see: Slominski *et al.*, 2000b; Bale & Vale, 2004; Charmandari *et al.*, 2005; Hillhouse & Grammatopoulos, 2006). In an addition α-MSH (Luger *et al.*, 1999; Bohm *et al.*, 2006; Eves *et al.*, 2006), intermediates of melanogenesis and melanin itself were found to affect immune response in the skin (Slominski *et al.*, 1998b; Slominski, 2009a). Moreover, ACTH (Hunt *et al.*, 1994; Abdel-Malek *et al.*, 1995; Luger *et al.*, 1999;

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Abbreviations: AC, adenylate cyclase; ACTH, adrenocorticotropic hormone; AP-1, activator protein 1; AtT-20, a pituitary adenoma cell line; COS-1 and COS-7, CARE, Ca^{2+} response element; CRE, cAMP response element; CREB, cAMP response element binding protein; CRF (CRH), corticotropin-releasing factor (hormone); CRF1 (CRH-R1, CRHR1, CRFR1), corticotropin-releasing factor receptor type 1; EC1-3, extracellular coil 1-3; ECD, extracellular domain (ligand binding domain); END, endorphin; G-protein, guanine nucleotide-binding protein; $G_{\alpha s}$ $G_{\alpha o}$, $G_{\alpha q/11}$, $G_{\alpha i}$ and $G_{\alpha z}$, G-protein subunits; GPCR, G-protein coupled receptor; HaCaT, immortalized human keratinocytes; HPA, hypothalamic-pituitary-adrenal axis; IC1-3, internal coil 1-3; IP_3, inositol trisphosphate; LPH, lipotropin; MSH, melanocyte stimulating homone; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; POMC, Pro-opiomelanocortin; SNP, single nucleotide polymorphism; SV40-transformed CV-1 kidney cell lines of African green monkey (*Cercopithecus aethiops*); 7TM, seven α-helical transmembrane domain; UCN-I, II, III, urocortins I, II and III; UV, ultraviolet.

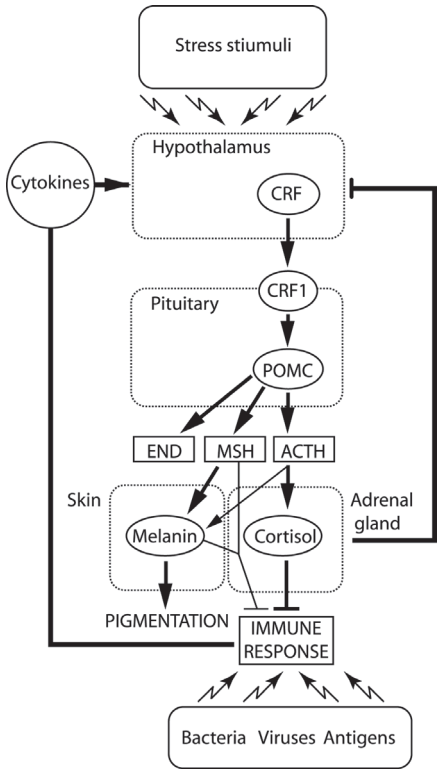

Figure 1. Mechanism of systemic response to stress through activation of the hypothalamic-pituitary-adrenal axis (HPA).
The signaling cascade is initiated by corticotropin releasing factor production (CRF) in the hypothalamus, followed by activation of the CRF receptor (CRF1) in the pituitary and subsequent synthesis of pro-opiomelanocortin (POMC). POMC-derived peptides: adrenocorticotropic hormone (ACTH), melanocortin (MSH) and endorphin (END), activate a series of key metabolic pathways including steroidogenesis in the adrenal gland (ACTH) and melanogenesis in the skin (MSH, ACTH and END). Synthesis and release of cortisol inhibits the immune response and form a negative feedback loop for CRF and POMC synthesis. In addition ACTH can trigger melanogenesis in the skin, and MSH, melanin and intermediates of melanogenesis suppress immune response. Modified after (Slominski *et al.*, 2000b). See text and citations within for details.

Slominski *et al.*, 2004b), and END (Kauser *et al.*, 2003) also stimulate melanogenesis in the skin. Thus, there is crosstalk between POMC derived peptides (Pawelek *et al.*, 1992; Slominski *et al.*, 1993; 2000b; Luger *et al.*, 1999).

The HPA axis is responsible for regulation of autonomic, behavioral, cardiovascular, endocrine, gastrointestinal, immune and reproductive functions on a central level in response to stress (see for extensive reviews: Chrousos, 1995; Slominski *et al.*, 2000b; Grammatopoulos & Chrousos, 2002; Hillhouse *et al.*, 2002; Tache & Bonaz, 2007). Acute or chronic stress and/or deregulation of the CRF system has been implicated in the pathophysiology of various psychological, neurological and endocrinological conditions, including anxiety, depression, abnormal pain and fatigue, sleep disorder (Hauger *et al.*, 2006; Clarke *et al.*, 2008; Wasserman *et al.*, 2008), drug and alcohol abuse (Clarke *et al.*, 2008), allergy, inflammation (Karalis *et al.*, 1991; Slominski *et al.*, 2001), gastrointestinal diseases (Tache & Bonaz, 2007), psoriasis (Arck & Paus, 2006; Slominski, 2009b) and acne (Ganceviciene *et al.*, 2009). Thus, CRF and its receptors are potential targets for therapy of several human conditions (Bale & Vale, 2004; Hemley *et al.*, 2007; Slominski *et al.*, 2008; Paschos *et al.*, 2009).

Recent studies revealed that the expression of the elements of the HPA-axis is not limited to the neuroendocrine system, but its elements are also found in immune cells (Slominski, 2006; Slominski *et al.*, 2000b), the digestive system (Tache & Bonaz, 2007), the skin and its appendages (Slominski & Mihm, 1996; Slominski *et al.*, 1996b; 2007a), the reproductive system (Hillhouse & Grammatopoulos, 2006) and many other tissues (Slominski *et al.*, 2000b; 2007b; Hillhouse & Grammatopoulos, 2006; Zmijewski *et al.*, 2007).

The peripheral expression of CRF, its receptors and other elements of the HPA axis are crucial in locally targeted regulation of the stress response, which is important in organs constantly subjected to stress insults like the skin (Slominski *et al.*, 1999; 2000b; Slominski & Wortsman, 2000; Arck *et al.*, 2006).

CRF RECEPTORS AND THEIR LIGANDS

CRF and its receptors play a central role in initiation and regulation of the central response to the stress (Spiess *et al.*, 1981; Vale *et al.*, 1981; Owens & Nemeroff, 1991; Charmandari *et al.*, 2005). CRF receptors belong to class B (secretin family) of G protein-coupled receptors (GPCRs), where the ligands are peptides of >27 amino-acid residues, produced mostly by endocrine cells (Perrin & Vale, 1999; Hillhouse & Grammatopoulos, 2006). Other members of class B are receptors for calcitonin, glucagon, glucagon-like hormone, secretin, parathyroid hormone (PTH), pituitary adenylate-cyclase-activating peptide, (PACAP), growth-hormone-releasing hormone (GHRH) (Miller *et al.*, 2007; Lagerstrom & Schioth, 2008).

Ligands

CRF is a 41-amino-acid peptide produced from the 18 kDa (21 kDa after posttranslational modification) precursor — pro-CRF (pro-CRH) (Spiess *et al.*, 1981; Vale *et al.*, 1981; Roche *et al.*, 1988). CRF is synthesized predominantly in the paraventricular nucleus of the hypothalamus, but it was also found in the peripheral tissues, including: reproductive organs (Asakura *et al.*, 1997; Clifton *et al.*, 1998), liver, stomach, spleen, adrenal and thyroid glands, bowel (Slominski *et al.*, 2000b; Charmandari *et al.*, 2005; Hillhouse & Grammatopoulos, 2006), retinal pigment epithelium (Zmijewski & Slominski, 2009a), kidneys (Slominski *et al.*, 2007b) and skin (Slominski *et al.*, 2001; 2006b). Its expression is stimulated by several neurotransmitters (serotonin, acetylcholine) (Ohmori *et al.*, 1995; Laflamme *et al.*, 1999), neuropeptides (arginine vasopressin, neuropeptide Y, enkephalin) (Itoi *et al.*, 1999) and cytokines (tumor necrosis factor (TNF), interleukins 1 and 6) (Turnbull & Rivier, 1999; Slominski, 2006). CRF is expressed in human skin (Slominski *et al.*, 1995; 1996a; 1998a; 2000a) and its expression is stimulated by external factors including ultraviolet (UV) irradiation, lipopolysaccharide (LPS) or factors raising cAMP level (Slominski *et al.*, 1996a; 1998a; 2006b; Zbytek & Slominski, 2007). An inhibitory effect on CRF production was shown for glucocorticoids, estrogens, dynorfin and substance P (reviewed in: Slominski *et al.*, 2000b). In addition to CRF, a family of CRF receptor ligands includes mammalian urocortins: UCN-I (urocortin 1), UCN-II (stresscopin-related peptide, urocortin 2) and UCN-III (stresscopin, urocortin 3); fish urotensin-I and sauvagine found in frog (Perrin & Vale, 1999; Slominski *et al.*, 2000b; Arck *et al.*, 2006; Hillhouse & Grammatopoulos, 2006; Hemley

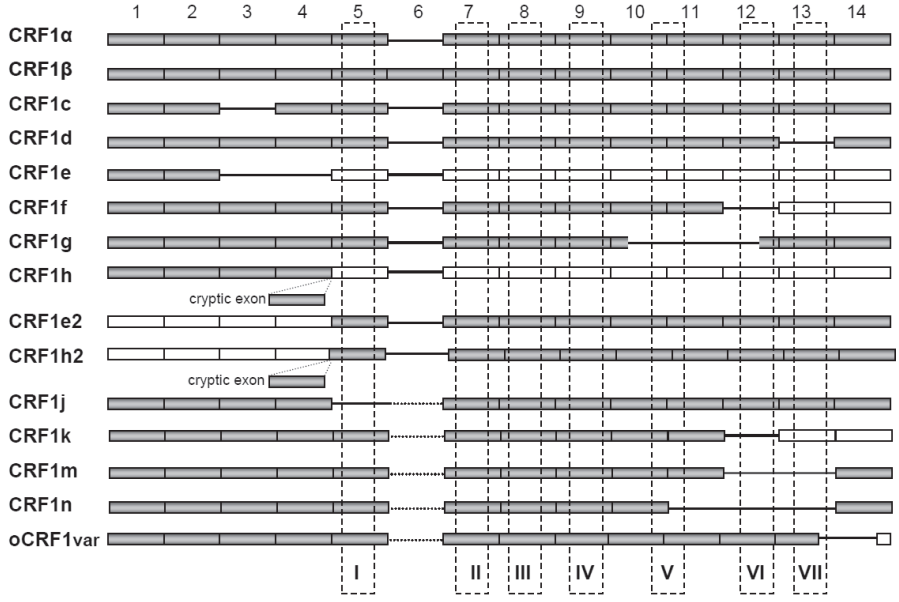

Figure 2. Schematic diagram of alternatively spliced isoforms of CRF1
CRF1 splicing variants differ in the number of exons (filed squares). Removed exons were shown as a line and exons with frame-shift as empty squares. Number of the exon (1 to 14) is indicated on the top. Names of CRF1 isoforms are indicated on the left. Isoforms α, β, c, d, e, f, g and h were detected in humans, mouse or hamster. CRF1e2 and CRF1h2 are theoretical alternative transcript from the same mRNA of CRF1e and h, respectively. Isoforms j, k, m and n were found only in hamster and isoform oCRF1var in sheep. Greek letters and doted boxes indicate transmembrane fragments I to VII. Modified after (Slominski *et al.*, 2006b).

et al., 2007). CRF-related peptides share about 50% of sequence homology and they show unique affinities to CRF receptors (Dautzenberg *et al.*, 2001).

CRF receptors

Human and all vertebrates express two types of CRF receptor — CRF1 and CRF2, also named: CRH-R1 and CRH-R2, or CRFR1 and CRFR2 (Perrin & Vale, 1999; Hauger *et al.*, 2006; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b). In the catfish a third member of the CRF receptor family was described as CRF3, which appears to be unique for this species (Arai *et al.*, 2001). The CRF1 and CRF2 share about 70% of homology among different species and CRF3 shows higher homology to CRF1 than CRF2. CRF receptors seems to have originated from one ancestor gene, which was duplicated (in the case of the catfish triplicated) in order to differentiate responsiveness to the selected ligands. Accordingly, CRF1 has high affinity for CRF and UCN-I, and very low for UCN-II, while CRF2 possesses high affinity for UCN-I, UCN-II and a lower one for CRF (Dautzenberg *et al.*, 2001; Hillhouse *et al.*, 2002; Bale & Vale, 2004; Hemley *et al.*, 2007). CRF3 bound CRF with a 5-fold higher affinity than urotensin-I and sauvagine (Arai *et al.*, 2001).

Although activation of CRF receptors is predominantly linked to up-regulation of cAMP through G_{sa} subunit and adenylate cyclase pathway, the phenotypic outcome is different depending on anatomic location or cell type. On the central level, stimulation of CRF1 results in production of ACTH leading to arousal, somatic nervous system activation and under chronic conditions causes depression. On the other hand, activation of CRF2 leads to appetite suppression and acts like an antidepressant. Thus, CRF1 is implicated in normal response to stress, while CRF2 meditates fine-tuning of the stress response. Moreover, two CRF receptors have opposite functions in regulation of behavior with CRF1 mediating anxiogenic and CRF2 anxiolytic effects (Hillhouse *et al.*, 2002; Bale & Vale, 2004).

In order to facilitate such diverse functions a unique and functionally relevant pattern of expression of CRF receptors was described in the brain (Van Pett *et al.*, 2000; Muller *et al.*, 2001; Bale & Vale, 2004; Hauger *et al.*, 2006). In the human skin, CRF1 was found in all major cellular populations including keratinocytes, melanocytes and dermal papilla fibroblasts, while expression of CRF2 was limited to adnexal structures including hair follicular keratinocytes and pigmentary unit, papilla fibroblasts, sebaceous and eccrine glands (Slominski *et al.*, 1995; 2000c; 2001; 2004a; 2007a; Pisarchik & Slominski, 2001; Zouboulis *et al.*, 2002; Ito *et al.*, 2004; Kauser *et al.*, 2006). Furthermore, expression of CRF receptor can be dynamic and change during development or be caused by physiological or pathological conditions (Slominski *et al.*, 2001; Slominski, 2003; Hillhouse & Grammatopoulos, 2006; Zmijewski & Slominski, 2009a).

ALTERNATIVE SPLICING OF CRF1 RECEPTOR REGULATES CRF SIGNALING

Alternative splicing is a very common feature among GPCR receptors, especially in the B family (including *Secretin* and *Adhesion* receptors). For instance, in airway smooth muscle, of 353 expressed GPCR — 192 (including CRF1) had, on average, five different splicing variants (Einstein *et al.*, 2008). Alternative splicing of CRF1 mRNA and function of isoforms in the brain structures and peripheral tissues including skin has been intensively studied during the last years (Chang *et al.*, 1993; Pisarchik & Slominski, 2001; 2002; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b; Nelson & Challiss, 2007; Einstein *et al.*, 2008). It was pos-

tulated that alternative splicing can explain differential responsiveness of receptors to agonists/antagonists in tissue- or cell-specific manner (Pisarchik & Slominski, 2001; 2004a; 2006b; Slominski, 2003; Hillhouse & Grammatopoulos, 2006).

Gene structure and properties of CRF1 receptor

The genomic sequence for human CRF1 (CRHR1) was mapped to chromosome 17 (17q12-q22) (Polymeropoulos *et al.*, 1995). The human CRF1 coding gene consists of 14 exons and 13 introns spanning about 20 kb and differs from the rat with 13 exons (Chen *et al.*, 1993; Tsai-Morris *et al.*, 1996; Sakai *et al.*, 1998; Parham *et al.*, 2004).

The general structure of CRF receptors is similar to other GPCRs and consists of an extracellular domain (ECD) or ligand binding domain encompassing the N-terminal fragment and three extracellular coils (EC1-3); a seven α-helical transmembrane domain (7TM) including three internal coils (IC1-3) and the C-terminus (Fig. 2 and 3). The most variable region of the CRF receptors is the N-terminal ECD with only 40% of homology between CRF1 and CRF2. The rest of the sequence is highly conserved with homology of 80% or greater for the 7TM domain and intracellular and extracellular loops. The third IC responsible for interaction with G-proteins was found to be identical for all CRFs (Hillhouse *et al.*, 2002; Hemley *et al.*, 2007).

The main isoform — CRF1α is a 415 amino-acid protein and is coded by 13 of 14 exons of CRF1 pre-mRNA (exon 6 is excluded). The first 23 amino acids form a signal peptide, which is subsequently processed (Perrin *et al.*, 2001). The structure of ECD of CRF1 could be predicted based on the solved NMR structure of CRF2β (Perrin *et al.*, 2006) and consists of two antiparallel β-sheets stabilized by three disulfide bonds between Cys30–Cys54, Cys44–Cys87 and Cys68–Cys102 forming a characteristic Sushi domain (Fig. 3). In addition, six or five N-glycosylation sides within the ECD of human or mouse CRF1 receptor were detected, respectively, but no O-glycosylation was observed (Assil & Abou-Samra, 2001; Hofmann *et al.*, 2001). PNGase F (peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase) treatment experiments additionally confirmed the presence of N-glycan residues (Sydow *et al.*, 1997; Slominski *et al.*, 2007b; Zmijewski & Slominski, 2009a). Glycosylation of CRF1 is thought to be responsible for proper function and trafficking of the receptor (Assil & Abou-Samra, 2001; Duvernay *et al.*, 2005).

Alternative splicing of CRF1 mRNA

In human, expression of at least eight splicing variants of CRF1 has been detected so far, and several other isoforms have been detected in other organisms or could be theoretically predicted (Fig. 2) (Pisarchik & Slominski, 2001; 2002; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b; 2007b).

Only one isoform, CRF1β, also called pro-CRH-R1 (Teli *et al.*, 2008) is coded by all 14 exon, because in the sequence of other isoforms exon 6 is spliced out. CRF1 splicing variants could be divided into three groups: full-length receptors (CRF1α, β), C-terminal, or ECD mutants (CRF1c and e) and isoforms with an impaired or missing 7TM domain (CRF1d, e, f, g and h). So-called "soluble" receptors (CRF1e, h) form a special subgroup of 7TM mutants lacking the entire 7TM domain. The main isoform CRF1α represents fully functional receptor and CRF1c has exon 3 spliced out. In isoform CRF1d exon 13 is missing. In the sequence of CRF1e exons: 3 and 4 are excluded, which causes a frameshift and introduction of a premature stop codon. CRF1f has exon 12 removed and in the sequence of CRF1g 27 base pairs of exon 10, the whole exon 11 and 28 base pairs of exon 12 are absent. CRF1h has an insertion of a cryptic exon between exons 4 and 5, resulting in a premature termination due to a frameshift (Pisarchik & Slominski, 2001; 2002; Slominski *et al.*, 2006b). In addition, mRNA of four unique isoforms (j, k, m and n) was detected in hamster (Pisarchik & Slominski, 2002). Isoform CRF1j has a deletion of exon 5 resulting in a frameshift in exon 6 (in rodents CRF1 has only 13 exons and there is no equivalent of human exon 6). Other isoforms named CRF1k, m and n have deletions within the 7TM domain as follows: exon 10; exon 11 and 12; or exons 10 to 12, respectively (Pisarchik & Slominski, 2002).

The list of CRF1 splicing variants could be extended with theoretical isoforms coded by CRF1e and CRF1h mRNAs. These two isoforms would have alternative ATG start codons and code sole 7TM domains (Fig. 3) (Pisarchik & Slominski, 2001; Slominski *et al.*, 2006b; 2007b). This prediction is additionally supported by detection of a "headless" isoform of calcitonin receptor — another member of family B of GPCRs (Nag *et al.*, 2007).

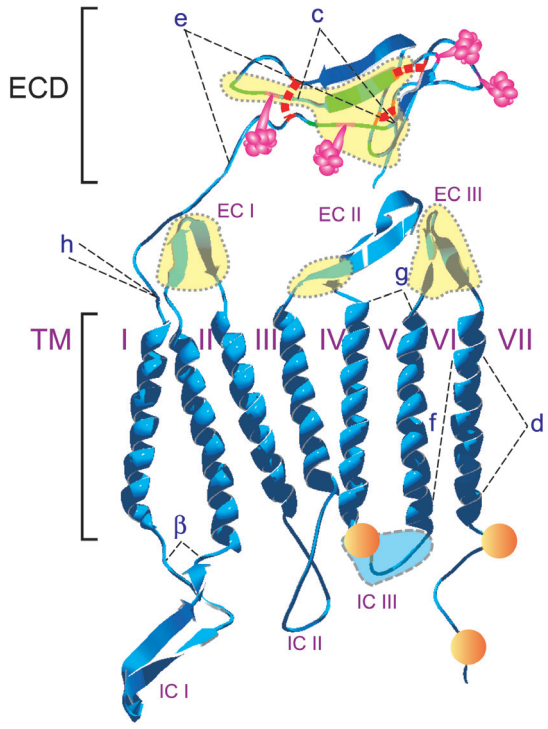

Figure 3. Model of CRF1 receptor and its splicing variants (α, β, c–h)
TM I–VII: transmembrane α-helical fragments, ECD: extracellular domain (ligand binding domain), EC I–III: extracellular coils, IC I–III: intracellular coils. Fragments removed by alternative splicing are shown by dotted lines. Fragments of ECD and EC III taking part in ligand binding are shown in yellow. Fragment of IC III responsible for G-protein binding shown in blue. Thick red dotted line: disulfide bonds, pink structures: glycosylation sides, orange balls: phosphorylation sides.

Expression of CRF1 isoforms

Alternative splicing of mRNA encoding CRF1 was detected in multiple tissues and organs including: the brain (Ross *et al.*, 1994), sheep pituitary (Myers *et al.*, 1998), human corticotropic tumor derived from Cushing syndrome patient (Chen *et al.*, 1993) and peripheral tissues including human skin (Pisarchik & Slominski, 2001; 2002; Slominski *et al.*, 2001; 2006a; 2006b; Zmijewski & Slominski, 2009a), myometrium (Hillhouse & Grammatopoulos, 2006; Jin *et al.*, 2007; Markovic *et al.*, 2007), adrenal glands (Sirianni *et al.*, 2005) retinal pigment epithelium (Zmijewski *et al.*, 2007), simian kidney cell line COS-7 (Slominski *et al.*, 2007b), human adrenal glands (Sirianni *et al.*, 2005), chorion trophoblasts (Gao *et al.*, 2007) and placenta (Karteris *et al.*, 1998). In addition hamster pituitary, eye, spleen, heart and skin was shown to express different CRF1 isoforms (Pisarchik & Slominski, 2002). The efficient expression of alternatively spliced isoforms of CRF1 was confirmed by Western blotting detection of multiple bands with different molecular weights (Grammatopoulos *et al.*, 1995; Sydow *et al.*, 1997; Rivier *et al.*, 2003; Pisarchik & Slominski, 2004; Slominski *et al.*, 2006a; 2007b; Zmijewski *et al.*, 2007). The relatively high level of variability in molecular weight and the number of CRF1 receptors detected in different tissues, could not be the result of sole methodological differences (sensitivity of antibodies) or posttranslational modification, like proteolysis or glycosylation (for discussion see: Sydow *et al.*, 1997; Slominski *et al.*, 2006a). A variety of experimental studies on CRF1 mRNA and protein level revealed that organs, tissues and cell lines produce unique patters of receptor isoforms due to alternative processing of pre-mRNA (Fig. 2).

Although the detailed mechanism of CRF1 splicing remains unknown, there is growing evidence that this process is affected by many factors including UV irradiation (Pisarchik & Slominski, 2001; Zmijewski & Slominski, 2009a), cyclic adenosine monophosphate (cAMP), phorbol 12-myristate 13-acetatate (PMA) (Pisarchik & Slominski, 2001), cell density (Zmijewski & Slominski, 2009a), pregnancy (Jin *et al.*, 2007) and onset of labor (Markovic *et al.*, 2007).

Recent studies have suggested that alternative splicing of CRF1 might be independent from regulation of receptor expression (Markovic *et al.*, 2007). Thus it can be proposed that splicing changes the pool of CRF1α mRNA. Interestingly, an induction of CRF1 splicing by cAMP might suggest the presence of a negative feedback, where an elevated concentration of cAMP due to stimulation of the CRF1α receptor would result in alternative splicing of the receptor, leading to at least partial inhibition of CRF signaling.

REGULATION OF CRF SIGNALING BY CRF1 ISOFORMS

Structure–function relationship plays a very important role in determination of activities of GPCRs and their isoforms. Detailed studies on CRF1 mutants and splicing variants revealed the functional importance for ECD and 7TM domains including external and internal coil regions and the C-terminus (Sydow *et al.*, 1997; Hofmann *et al.*, 2001; Perrin *et al.*, 2001; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b; Markovic *et al.*, 2008; Zmijewski & Slominski, 2009a). As predicted, expression of ECD, alone, was sufficient for ligand binding, but the binding was enhanced by the presence of extracellular loops (Sydow *et al.*, 1997). The 7TM domain is essential for proper signal transduction, resulting in activation of G-proteins and subsequent production of second messengers (cAMP, IP_3 and Ca^{+2}). IC-3 was shown to be involved in G-protein interaction and the presence of the C-terminus enhanced cAMP production (Grammatopoulos *et al.*, 1999; Pisarchik & Slominski, 2004). In addition to structural properties of CRF1 isoforms (and other GPCRs), their activity depends on their cellular localization. Specifically, CRF1 isoforms can be divided into three categories: membrane-bound, intracellular and extracellular (soluble) receptor forms (Slominski *et al.*, 2006b).

It should be noted that a change in the receptor sequence by alternative splicing often results in frameshift and is responsible for premature termination of transcription. Such transcripts are prone to activate the nonsense-mediated mRNA decay mechanism (Lewis *et al.*, 2003). Nevertheless, recent advances in receptor physiology suggest that these mRNA might encode proteins with modified but functional properties (Pisarchik & Slominski, 2004; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b; Zmijewski & Slominski, 2009a; 2009b).

Classical pathway — CRF1α

CRF1α is the main and fully functional isoform of the receptor and the majority of publications concerning the CRF1 receptor (CRFR1, CRHR1, CRH-R1) refer to this isoform.

In the classical pathway, ligand binding to CRF1 leads to signal transduction across the cell membrane resulting in activation of heterotrimeric G-proteins (Fig. 4). Detailed studies have revealed that the CRF receptor interacts with several G-proteins with preference for $G_{\alpha s}$ and lower affinity for $G_{\alpha o}$, $G_{\alpha q/11}$, $G_{\alpha i}$ and $G_{\alpha z}$ (Grammatopoulos *et al.*, 1999). Although, the primary target is represented by the activation of $G_{\alpha s}$ (adenylate cyclase (AC) – protein kinase A (PKA) pathway), it has to be noted that activation of specific G-proteins and signaling pathways can be tissue and cell type specific (Slominski *et al.*, 2006a). It remains to be investigated if expression of alternative isoforms of CRF1 is responsible for modulation of affinity for G-proteins or whether this process is regulated by other mechanisms including, e.g., phosphorylation of the receptor.

The increase in concentration of secondary messengers (cAMP, IP_3 and Ca^{2+}) in cells triggered by CRF1α ligands, results in activation of multiple transcriptional factors including CREB, AP-1, NF-κB, and the calcium response element (CARE) (Fig. 4) (Fazal *et al.*, 1998; Wiesner *et al.*, 2003; Pisarchik & Slominski, 2004; Zbytek *et al.*, 2004; 2005; Zbytek & Slominski, 2005; Slominski *et al.*, 2006b; Zmijewski & Slominski, 2009a; 2009b). The CRE element was shown to be stimulated through the signaling cascade involving $G_{\alpha s}$-mediated activation of adenylate cyclase (AC), cAMP accumulation, PKA activation and CREB phosphorylation at Ser-133 (Fig. 4) (Pisarchik & Slominski, 2001; Eckart *et al.*, 2002; Slominski *et al.*, 2006b). CRF stimulation also results in $G_{\alpha q}$-protein dependent activation of phospholipase C (PLC), leading to IP_3 accumulation. Subsequent activation of PKC results in an indirect stimulation of expression and accumulation of AP-1 transcription factor in the nucleus (Hofmann *et al.*, 2001; Eckart *et al.*, 2002; Pisarchik & Slominski, 2004; Slominski *et al.*, 2006b). CRF and urocortin stimulation in the skin results in an increase of intracellular Ca^{2+} concentration *via* a dual mechanism which involves release of Ca^{2+} from intracel-

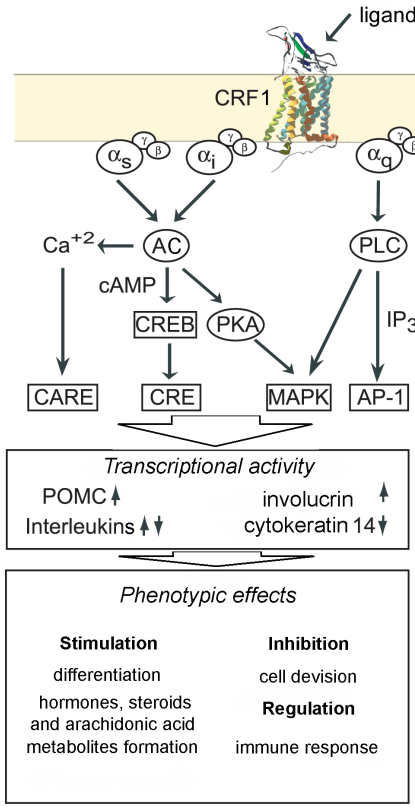

Figure 4. Activation of intracellular signaling pathways by stimulation of CRF1 receptor
Ligand binding to CRF1 receptor activates at least three G_α subunits (α_s, α_i and α_q) of G-protein resulting in stimulation cAMP production by adenylate cyclase (AC) with subsequent cAMP-induced phosphorylation of CREB and activation of the cAMP-responsive element (CRE). Mobilization of calcium (Ca^{+2}) results in activation of the calcium-responsive element (CARE). Activation of AP-1 can also trigger the mitogen-activated protein kinase (MAPK) pathway through protein kinase A (PKA). In addition, α_q-mediated stimulation of phospholipase C results in IP_3-driven activation of activator protein 1 (AP-1) dependent promoters. Downstream signaling from CRF1 receptor regulates expression of several genes including POMC, several interleukins, involucrine and cytokeratin 14. The phenotypic effects of stimulation include: stimulation of differentiation, steroidogenesis, melanogenesis and release of arachidonic acid. CRF inhibits cell division and regulates immune response. See text and citations within for details.

lular stores and increases Ca^{2+} influx by the opening of voltage-activated Ca^{2+} ion channels (inhibited by EGTA, d-*cis*-diltiazem and verapamil) (Fazal *et al.*, 1998; Slominski *et al.*, 1999; Wiesner *et al.*, 2003). CRF can also activate CARE and this activation is mediated thought the CRF1α isoform as shown in HaCaT keratinocytes (Pisarchik & Slominski, 2001; Zmijewski & Slominski, 2009a), COS-7 cells (Slominski *et al.*, 2007b) or AtT-20 cells (Zmijewski & Slominski, 2009b).

The activation of multiple signaling pathways is responsible for several phenotypic manifestations of CRF1α stimulation. Besides classic stimulation of POMC expression (Fig. 1) activation of CRF1α receptor influences cell proliferation and differentiation (Fig. 4) (Slominski *et al.*, 2000c; 2006a; 2006b; Kauser *et al.*, 2006; Conn *et al.*, 2007; Ganceviciene *et al.*, 2009). Moreover, CRF and its receptors are important for immunomodulation (Karalis *et al.*, 1991; Slominski, 2003; Theoharides *et al.*, 2004; Arck *et al.*, 2006; Slominski *et al.*, 2006a; Zbytek & Slominski, 2007; Paschos *et al.*, 2009). Differentiation program of the keratinocytes has been linked to activation of AP-1 family members such as JunB, JunD and Fra1 (Eckert *et al.*, 2004; Zbytek *et al.*, 2005; Zbytek & Slominski, 2005). Activation of CRF1 receptor modulates the activity of NF-κB — the main regulator of cell viability, differentiation and cytokine production (Zbytek *et al.*, 2003; 2004; 2006a; Slominski *et al.*, 2006b). This process was studied in-depth using epidermal skin cells (Zbytek *et al.*, 2003; 2004; 2006a) and it was postulated that CRF and its receptor play a dual role in regulation of NF-κB activity depending on the prevailing environmental context (Paez Pereda *et al.*, 1995; Slominski, 2003; Slominski *et al.*, 2006b). Depending on conditions but according to the classic model (Baeuerle & Baltimore, 1988) activation of CRF1α receptor in the skin influences stability of IκB-α, which serves as a negative regulator of NF-κB (Slominski, 2003; Zbytek *et al.*, 2003; 2004; Slominski *et al.*, 2006b). Other investigators also reported, either stimulatory or inhibitory effects of CRF on immunocompetent skin cells (Quevedo *et al.,* 2001; Zbytek *et al.*, 2003; 2004), and CRF-dependent regulation of NF-κB activity in different experimental models (Lezoualc'h *et al.*, 2000; Zhao & Karalis, 2002). CRF-driven activation of NF-κB pathway was also observed in normal keratinocytes (Zbytek *et al.*, 2004) but in contrast stimulation of normal melanocytes with CRF had the inhibitory effect on the NF-κB activity (Zbytek *et al.*, 2006a).

Thus, the activation of secondary messengers and phenotypic effects of CRF1α stimulation is cell type-dependent and regulated by external and internal factors. It is tempting to conclude that such a diversity is regulated at least in part by expression of several CRF1 isoforms. Moreover, recent studies have revealed that alternative splicing is a dynamic process of adaptation to the changing environment (Markovic *et al.*, 2007; Zmijewski & Slominski, 2009a). Unfortunately, there is a shortage of information on the phenotypic consequences of alternative splicing of CRF1 which might lead to wrong assumption concerning CRF signaling in given cellular model.

Membrane-bound isoform — CRF1β

CRF1β is sometimes referred to as pro-CRF1, because it is encoded by all the 14 exons, including exon 6 spliced out from all the other known isoforms (Chen *et al.*, 1993; Xiong *et al.*, 1995). Integration of the 29-amino-acid fragment encoded by exon 6 into first intracellular coil of CRF1 only slightly inhibits substrate-binding properties of the receptor (2.1×), but cAMP production was found to be impaired (100× decrease) (Xiong *et al.*, 1995). Recent studies have shown that a positively charged fragment (F170–R174) of the CRF1β insert is responsible for inhibition of cAMP response (Teli *et al.*, 2008). Furthermore, retention of exon 6 also increases responsiveness to PKC-induced phosphorylation and results in premature desensitization of signaling and internalization of the receptor (Markovic *et al.*, 2006). Thus, despite of proper cell membrane localization CRF1β and possession of all the structural features of the native receptor, CRF signaling through CRF1β is impaired.

Membrane bound isoform — CRF1c

CRF1c, the other example of a CRF1 isoform possesses an intact 7TM domain (Ross *et al.*, 1994), which indicates proper cell membrane localization (Slominski *et al.*, 2006b), and this has been recently confirmed experimentally (Zmijewski & Slominski, 2009a; 2009b). On the other hand, a deletion of 40 amino acids encoded by exon 3 removes the central part of ECD including

three out of six cysteines. These residues are crucial in formation of disulfide bridges essential for stability of the ECD domain (Fig. 3). Thus, in spite of the proper membrane localization, CRF1c expressed in the kidney cell line COS-1, failed to bind agonists (^{125}I-oCRF) (Ross *et al.*, 1994). Elevation of the intracellular level of cAMP was observed only after stimulation with a high concentration of human CRF (Ross *et al.*, 1994; Grammatopoulos *et al.*, 2000). It has to be noted that detection of CRF stimulated accumulation of cAMP in cells overexpressing CRF1c could be at least partially explained by endogenous expression of CRF1 shown in the another fibroblast-like kidney cell line COS-7 (Slominski *et al.*, 2007b). Moreover, CRF and urocortin were not able to stimulate CRF1c mediated IP_3 production, MAPK phosphorylation or CRE activation in human embryonic kidney line (HEK 293) overexpressing CRF1c (Grammatopoulos *et al.*, 2000; Markovic *et al.*, 2008).

CRF1 isoforms with impaired 7TM domain

At least 5 human isoforms of CRF1 receptor (CRF1d, e, f, g and h) have alterations within the 7TM domain (Pisarchik & Slominski, 2001; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b). In hamster, in addition to human-like isoforms CRF1e, f and h, mRNAs of 4 unique CRF1 splicing variants (CRF1j, k, l and m) were detected (Pisarchik & Slominski, 2002). 7TM variants represent variety of single or multiple exon deletions with or without frameshift (Fig. 2) (Pisarchik & Slominski, 2002; Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b). In addition, a CRF1 isoform named oCRF1var with a deletion of 34 amino acids forming the last α-helical fragment of 7TM domain and a part of the C-terminus was described in sheep (Myers *et al.*, 1998). Additional, so called "soluble" isoforms (CRF1e, j and h) lack the entire 7TM domain and will be described in the next chapter.

CRF1d with a characteristic deletion of a 14-amino-acid fragment of the 7TM domain coded by exon 12, was first described by the Gramatopoulos group (Grammatopoulos *et al.*, 1999; Hillhouse & Grammatopoulos, 2006). The deletion of a fragment of the seventh transmembrane α-helix did not result in a decrease in substrate binding affinity, when compared with CRF1α. However, distortion of the 7TM domain inhibits interaction of the receptor with G-proteins ($G_{\alpha s}$, $G_{\alpha o}$, $G_{\alpha q}$ and $G_{\alpha i}$) and results in subsequent inhibition of downstream signaling, as judged by an at least 10-fold decrease in cAMP synthesis and total inhibition of IP_3 production (Grammatopoulos *et al.*, 1999). As a consequence, inhibition of activation of cAMP or IP_3 response elements (CRE and AP-1) was observed in cells overexpressing CRF1d (Zmijewski & Slominski, 2009a; 2009b). In SKMEL-188 melanoma cells, which only express the CRF1d isoform, the lack of coupling to cAMP with overt stimulation of Ca^{2+} flux suggest that the CRF1d signal transduction pathway is only coupled to either voltage-activated Ca^{2+} ion channels or PLC (Slominski *et al.*, 2006a; Zmijewski & Slominski, 2009b). Ovine CRF1var was also shown to bind oCRF (ovine CRF) with comparable affinity to the native receptor, but oCRF-stimulated accumulation of cAMP and internalization of the receptor was impaired (Myers *et al.*, 1998). Inhibition of downstream signaling as measured by cAMP accumulation or activation of CRE, CARE, AP-1 transcription elements was also observed in various cells overexpressing human isoforms CRF1f and g (Pisarchik & Slominski, 2004; Zmijewski & Slominski, 2009a; 2009b). Thus, it is postulated that distortion within the 7TM domain resulting from alternative splicing of CRF1 mRNA does not significantly influence an affinity for ligands, but affects binding to G-protein and, consequently downstream signaling through the receptor is impaired.

Recent studies have shown that overexpression of CRF1d in contrast to CRF1α resulted in intracellular accumulation of this 7TM mutant (Markovic *et al.*, 2008; Zmijewski & Slominski, 2009a; 2009b). Intracellularly, CRF1d was found to be associated with the endoplasmic reticulum and CRF1f and g with Golgi cisterns in human HaCaT keratinocytes (Zmijewski & Slominski, 2009a). These findings might explain high intracellular CRF1 immunoreactivity in human skin tissue and cell lines or uterine smooth muscle cells, known to express multiple CRF1 isoforms including 7TM mutants (Slominski *et al.*, 2006a; Markovic *et al.*, 2007; Zmijewski & Slominski, 2009a).

A recent study by Markovic and collaborators (Markovic *et al.*, 2008) showed that cassette G356–F358 within the seventh transmembrane helix of CRF1α (exon 13) is crucial for membrane localization of the receptor (Figs. 2 and 3). Deletion or misplacement of this fragment is characteristic for all 7TM isoforms of CRF1 (Figs. 2 and 3). Interestingly, CRF1d and a calcitonin receptor splicing variant (CRTDe13) have an analogous exclusion of exon 13 with similar physiological consequences (Shyu *et al.*, 1996; Seck *et al.*, 2003; 2005). Moreover, co-expression of CRF1α with CRF1d, f or g results in retention of both isoforms inside the cell (Zmijewski & Slominski, 2009a; 2009b). Intracellular retention of CRF1 isoforms is most likely associated with their oligomerization (Kraetke *et al.*, 2005; Mikhailova *et al.*, 2007; Markovic *et al.*, 2008; Zmijewski & Slominski, 2009a; 2009b). Thus, it could be postulated that distortion of the 7TM domain of CRF1 receptor (and other GPCRs) results in at least partial retention of the reception in the cell, impairs G-protein binding and signal transduction despite only slightly altered ligand binding. In addition an intracellular localization sequesters the receptor and its homo- and heterodimers prevents an interaction with extracellular substrates.

Soluble isoforms

At least two human, mouse and three hamster CRF1 isoforms lack entire 7TM domains, due to alternative splicing (Pisarchik & Slominski, 2001; 2002). In an analogy to artificial ECD models: mNT-CRF1 and sCRFR2α, they are called "soluble" forms (Perrin *et al.*, 2001; Pisarchik & Slominski, 2001; Chen *et al.*, 2005).

CRF1h (human, mouse and hamster) similarly to CRF1j (only found in hamster) are encoded by exons 1–4 of full length CRF1. Deletion of exon 5 results in a frameshift and introduction of a premature stop codon. In an addition CRF1h is encoded by a cryptic exon immediately after exon 4 (Pisarchik & Slominski, 2001; Slominski *et al.*, 2006b). Similarly to artificial constructs — mNT-CRFR1 (Perrin *et al.*, 2001) and sCRF2α (Chen *et al.*, 2005) the soluble isoform CRF1h may be capable of binding the ligands. This is in contrast to CRF1e isoform missing most of its ECD (Figs. 2 and 3) (Slominski *et al.*, 2006b). Although activity of different artificial models of ECD was proven, their ability to bind substrates was found to be lower when compared to full length receptor. For instance, rat rCRFR1-NT-Kif construct (rat model of ECD), specifically bound to arrestin and rat UCN, but the affinity was found to be 10-folds lower than for full-length rat CRF1 (Hofmann *et*

al., 2001). Similarly, binding of several ligands (rUCN-1, mUCN-2, mUCN-3, r/hCRF) to the ECD1-CRFR2β construct (containing ECD of CRF2β receptor) was 5 to 10 times lower when compared to full length CRF2β expressed in Chinese hamster ovary cell line (CHO). The same study revealed that ECD1-CRFR2β soluble receptor was not able to bind sauvagine, suggesting that another part of CRF2 receptor was specifically responsible for sauvagine binding (Perrin *et al.*, 2003). Other experiments showed involvement of extracellular coil EC-3 in ligand binding and this finding might partially explain lower substrate affinity of soluble CRF receptor isoforms containing exclusively ECD (Sydow *et al.*, 1997; Gkountelias *et al.*, 2009).

Overexpression of CRF1h in immortalized human HaCaT keratinocytes results in co-localization with endoplasmic reticulum and cytoplasm, but not with cell membrane or CRF1α (Zmijewski & Slominski, 2009a). Interestingly, both CRF1h and to a lower degree CRF1e (Zmijewski & Slominski, 2009a), similarly to mNT-CRFR1 (Perrin *et al.*, 2001) and sCRF2α (Chen *et al.*, 2005), could be released to the medium. Thus, localization of soluble isoforms might determine their function. Co-expression of CRF1h with CRF1α increased in production of cAMP in COS-7 cells (Pisarchik & Slominski, 2004), but caused decrease or had no influence on the activity of cAMP or AP-1 responsive elements in HaCaT keratinocytes (Zmijewski & Slominski, 2009a) and AtT-20 pituitary cells (Zmijewski & Slominski, 2009b), suggesting involvement of other CRF1 isoforms or/and cell specific factors. Potential activity of secreted soluble isoforms was shown in a "media exchange" experiment performed on AtT-20 cells overexpressing CRF1 isoforms. Presence of CRF1h and to a lesser extend CRF1e in culture medium partially inhibited stimulation of CRF1α by CRF in AtT-20 corticotrophic cells (Zmijewski & Slominski, 2009b). Thus these isoforms, under certain conditions, could act as soluble decoy receptors, as previously suggested (Pisarchik & Slominski, 2004; Chen *et al.*, 2005).

CRF1e protein has only 40 amino acids of native protein including a signal peptide of 23 amino acids. The major part of ECD responsible for ligand binding is missing and the remaining 104 amino acids incorporated to the sequence due to frameshift have no homology to any known protein (Pisarchik & Slominski, 2001). In addition, it seems that CRF1e mRNA, or/and protein is not stable because GFP signal from fusion receptor CRF1e-GFP fades away a few days after transfection, while overexpression of other fusion constructs containing CRF1 isoforms resulted in stable fluorescence for more than a week (Zmijewski & Slominski, 2009a). CRF1e was equally distributed within the cells when overexpressed in HaCaT keratinocytes or AtT-20 pituitary cells. In addition, CRF1e was the sole receptor isoform found in the nucleus. Co-expression of CRF1e and CRF1α did not result in co-localization of both isoforms, but unexpected intracellular aggregation of CRF1α was observed (Zmijewski & Slominski, 2009a). Further studies are necessary to establish a mechanism for those phenomena. Transient transfection with CRF1e did not result in activation of CRF-driven signaling in cells of various origins (Pisarchik & Slominski, 2004; Zmijewski & Slominski, 2009a; 2009b). It is possible that accumulation of CRF1e mRNA can lower the pull of CRF1α mRNA spliced out from common precursor, having a negative effect on final CRF1α expression. This hypothesis requires additional experimental validation.

ROLE OF ALTERNATIVE SPLICING

It has been widely accepted that the expression of certain types of GPCR receptors does not fully explain the variety of cellular responses after stimulation with selective agonists. Many factors could be involved in such regulation, including posttranslational modification, receptor oligomerization, constitutive activation or presence of cell specific modifiers (Nelson & Challiss, 2007; Zmijewski & Slominski, 2009a).

Alternative splicing is a common feature of at least 70% of human genes being responsible for increased functional capacity of the human genome. Many GPCRs including CRF1 and other members of the B1 family (secretins) have several variants generated by alternative splicing (Einstein *et al.*, 2008). Although the detailed mechanism of alternative splicing has been studied for many years, its function is not well understood, especially in the case of GPCRs (Minneman, 2001; Bjarnadottir *et al.*, 2007).

Expression of the majority of alternative spliced variants of CRF1 results in a dominant negative phenotype, or at least modulatory responses (Fig. 5). Moreover, alternative splicing of CRF1 is not only tissue- or organ-specific, but it is also tightly regulated by intra and extracellular factors (Hillhouse & Grammatopoulos, 2006; Slominski *et al.*, 2006b; Zmijewski & Slominski, 2009a). In addition, alternatively spliced mRNA of CRF1 isoforms may undergo fast mRNA decay process (nonsense-mediated decay — NMD). This process may be linked to the presence of premature termination codon introduced by alternative splicing (Lewis *et al.*, 2003; Amrani *et al.*, 2006). Several CFR1 isoforms including CRF1e, f, h and k, possess alternative, premature stop codons introduced by alternative splicing (Fig. 2). It seems unlikely that such a complex system, conserved in evolution is just a random noise of the spliceosome.

There is growing evidence that alternative splicing of CRF1 pre-mRNA is responsible for modulation of CRF signaling. In the current model, isoform CRF1α has a dominant role as a fully functional receptor and the remaining isoforms regulate its activity (Fig. 5). The first and obvious consequence of alternative splicing of CRF1 mRNA is lowering of the pool of mRNA of native receptor (CRF1α), which may potentially inhibit of CRF signaling. Another function of CRF1 isoforms may be formation of receptor oligomers (dimers) (Sirianni *et al.*, 2005; Gurevich & Gurevich, 2008). Although dimerization does not appear to be essential for ligand binding (Kraetke *et al.*, 2005), CRF1 was found to form homo- and heterooligomers by several groups (Kraetke *et al.*, 2005; Mikhailova *et al.*, 2007; Young *et al.*, 2007; Zmijewski & Slominski, 2009a). High molecular complexes containing CRF1 isoforms were stable under mild denaturating conditions during SDS/PAGE separation (Sydow *et al.*, 1997; Pisarchik & Slominski, 2004; Slominski *et al.*, 2007b; Zmijewski *et al.*, 2007; Zmijewski & Slominski, 2009a). Interestingly, such complexes are sensitive to deglycosylation and reducing agents (dithiothreitol) suggesting a role of N-glycans and disulfide bonds in the stability of CRF1 ternary complexes (Zmijewski & Slominski, 2009a). The formation of homo- and heterodimers of CRF1 isoforms might also regulate of CRF1α localization and activity. For example, heterooligomerization could explain intracellular co-localization of CRF1α with isoforms with impaired 7TM

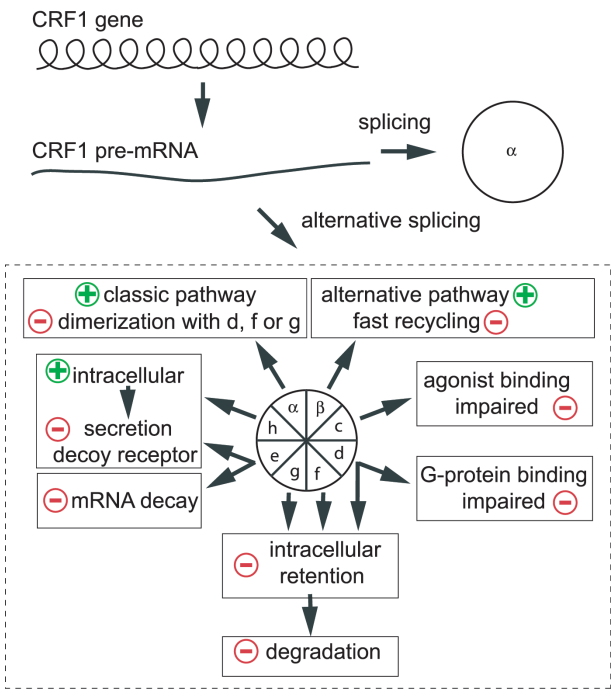

Figure 5. Regulation of CRF signaling by CRF1 isoforms
CRF1 gene contains 14 exons and only one isoform of receptor–CRF1β (also called pro-CRF1) is coded by all exons. CRF1 transcript is also subjected to alternative splicing resulting in at least 8 isoforms. Recent studies showed that expression and/or co-expression of CRF1 isoforms is responsible for modulation of CRF1 signaling. Plus indicates stimulation of downsteam signaling by the classic pathway (CRF1α) or alternative pathway (CRF1β). Soluble isoforms (CRF1e and h) were also found to stimulate CRF signaling when co-expressed with CRF1α. Minus indicates inhibition of CRF signaling on different levels including**:** fast mRNA decay (CRF1e), dimerization and subsequent intercellular retention resulting in most probable premature receptor degradation (CRF1α with CRF1d, CRFf or CRFg), decoy receptor mechanism (CRF1h and e when secreted), agonist binding impairment (CRF1c) or G-protein wbinding inhibition (CRF1d). See text for details and citations.

domains (CRF1d, f and g) and resulting partial inhibition of CRF signaling. Recently a similar interaction was observed for two isoforms of CRF2, e.g., CRF2β, and (iv)-mCRF2β (Sztainberg *et al.*, 2009). Moreover, CRF1β was found to be rapidly internalized after formation of heterodimers with CRF1α. Therefore, we postulated that heterooligomerization of CRF1 isoforms can diversify the repertoire of functional CRF1 receptors and/or modulate CRF signaling (Slominski *et al.*, 2006b; Markovic *et al.*, 2008; Zmijewski & Slominski, 2009a; 2009b).

Soluble isoforms of CRF1 (CRF1e, h) are similar to mNT-CRFR1 (Perrin *et al.*, 2001) and sCRF2α (Chen *et al.*, 2005) and their biological activities have already been demonstrated. The mechanism of extracellular activity of soluble isoforms should be similar to CRF binding protein (CRF-BP), which serves as an extracellular binding protein that resembles decoy receptors (Chen *et al.*, 2005).

It must be also mentioned that the activity of the receptors from class B of GPCR can be modulated by several other factors, including receptor activity-modifying proteins (RAMPs) (Sexton *et al.*, 2006), β-arrestins (Holmes *et al.*, 2006), G protein-coupled receptor kinases (GRK1-3) or Rab GTPases (Miller *et al.*, 2007).

CRF1 IN HUMAN DISEASES, ALTERNATIVE SPLICING AS A POSSIBLE TARGET

GPCRs are a target for 40–50% of all marketed drugs (Hemley *et al.*, 2007) and CRF1 antagonists and agonists have potential benefits in the therapy of several human pathologies. The agonists of CRF1 receptor with low hypotensive activity such as [D-Glu20]-CRH may be used in therapy of malignant melanoma as their antiproliferative activity was shown *in vivo* and in an animal model (Carlson *et al.*, 2001). Human and rodent skin cancer cell lines including melanomas (Pisarchik & Slominski, 2001; 2002), squamous cell carcinomas and basal cell carcinomas (Pisarchik & Slominski, 2001) express different patterns of CRF1 isoforms, thus alternative splicing of CRF1 may play an important role in sensitivity of cancer cells to CRF and its analogues. In general skin cancer cell lines express multiple isoforms of CRF1 receptor. It has to be stressed that the effect of CRF and CRF-related peptides strongly depends on cellular context as has been shown for different skin cells (Zbytek *et al.*, 2003; 2004; 2005; 2006a; 2006b; Slominski *et al.*, 1999; 2000c; 2006b; 2007a).

Single nucleotide polymorphism (SNP) another factor, which might also influence alternative splicing of CRF1 mRNA. This phenomenon is of particular interest, because CRF1 SNPs have been associated with many human conditions, including: depression (Liu *et al.*, 2006), stress, alcohol abuse (Blomeyer *et al.*, 2008; Schmid *et al.*, 2009), suicidal tendencies (Wasserman *et al.*, 2008), infection susceptibility, asthma (Lima *et al.*, 2009) and bone density (Jones *et al.*, 2008). Thus, in-depth investigation of CFR1 splicing in the context of CRF1 SNPs might bring some mechanistic explanation for genetically determined changes in CRF1 receptor activity.

Several antagonists of CRF1 receptors have been studied including CRF analogues (α-helical CRF and other compounds see: Wei & Thomas, 1994; Rivier *et al.*, 2002; 2007) and antalarmin (non-peptide CRF1 antagonist) (Webster *et al.*, 1996; Deak *et al.*, 1999) as well several other agonists and antagonists of CRF1 and CRF2 (see for review: Gulyas *et al.*, 1995; Reyes *et al.*, 2001). Antalarmin and its derivatives, as the potent CRF1 antagonists have been successfully used in animal models of anxiety disorders, alcohol and drug addiction (Zoumakis *et al.*, 2006), inflammatory bowel syndrome (Paschos *et al.*, 2009).

Certaily, detailed studies on CRF1 alternative splicing and possible influence of local environment are required in order to assess potential benefits of CRF1 agonist/antagonist therapy (Wei & Slominski, 2002; Vale, 2004; Pisarchik & Slominski, 2006). On the other hand modulation of CRF1 activity by an alternative splicing may represent a promising strategy to regulate sensitivity of cells to CRF1 agonists or antagonists. There are several factors and drugs with proven influence on alternative splicing (for review see: Sumanasekera *et al.*, 2008). Surprisingly, many anticancer drugs including daunorubicin and cisplatin can also affect alternative splicing as shown for *Bcl-X* and other human apoptotic genes (Shkreta *et al.*, 2008). Thus, selective modulation of CRF1 splicing is feasible. For example, splice-switching oligonucleotides (for recent review see: Bauman *et al.*, 2009) or chemically modified antisense oligonucleotides (Kole *et al.*, 2004) could be used to change the pattern of expression of CRF1 isoforms in order to sensitize or desensitize cells to CRF1 agonists. Specifically, induction of CRF1 variants c, d, f or g by CRF1-targeted splice-switching

oligonucleotides might be alternative to antalarmin in treatment of anxiety disorders, alcohol and drug addiction, inflammatory bowel syndrome. On the other hand, it is possible that directing alternative splicing towards expression of the CRF1α isoform may sensitize cells to CRF-driven inhibition of cancer cell proliferation (for example, melanoma). Thus, induced modulation of alternative splicing of CRF1 may represent a dawn of new therapeutic strategies targeting a variety of diseases (Pisarchik & Slominski, 2006).

CONCLUSION

There is growing evidence that alternative splicing of CRF1 receptor represents an additional level of regulation of central and local responses to stress *via* the HPA axis. Expression of several CRF1 isoforms depends on a variety of external and internal factors and appears to be responsible for regulation of activity of CRF signaling. Thus, the alternative splicing of CRF1 (and other GPCRs) is not only a very important and complex regulatory mechanism but may also represent a novel pharmacological target in therapy of various disorders (Wei & Slominski, 2002; Hillhouse & Grammatopoulos, 2006; Pisarchik & Slominski, 2006; Slominski *et al*., 2006b; Einstein *et al*., 2008; Wasserman *et al*., 2008; Lima *et al*., 2009; Sztainberg *et al*., 2009; Zmijewski & Slominski, 2009a).

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