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# MODULATION OF CORTICOTROPIN RELEASING FACTOR (CRF) SIGNALING THROUGH RECEPTOR SPLICING IN MOUSE PITUITARY CELL LINE ATT-20 - EMERGING ROLE OF SOLUBLE ISOFORMS

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Previously, using cultured human epidermal keratinocytes we have demonstrated that the activity of CRF1 receptor can be modulated by the process of alternative splicing. This phenomenon has been further investigated in the mouse corticotroph AtT-20 cell line. In the cells, transiently transfected with the plasmids coding human CRF1 isoforms, only isoforms α and c have shown expression on the cell membrane. Other isoforms d, e, g and h had intracellular localization with the isoform e also found in the nucleus. Co-expression of the CRF1α (main form of the receptor) with isoforms d, f and g prevented its expression on the cell surface resulting in accumulation of CRF1α inside of the cell. As expected, CRF stimulated time and dose dependent activation of CRE, CARE, AP-1 transcription elements and POMC promoter in AtT-20 cells overexpressing human CRF1α, while having no effect on the AP-1 transcriptional activity in cells transfected with other isoforms (d, f, g and h). However, when cells were co-transfected with CRF1α and CRF1e or h the CRF stimulated transcriptional activity of CRE and AP-1 was amplified in comparison to the cells expressing solely CRF1α; the effect was more pronounced for CRF1h than for CRF1e. In contrast, the conditioned media from the cells overexpressing CRF1e and h inhibited the CRF induced transcriptional activity in cells overexpressing CRF1α. Media from cells expressing CRF1h were significantly more potent that from cells transfected with CRF1e. In summary, we have demonstrated that alternatively spliced CRF1 isoforms can regulate the cellular localization of CRF1α, and that soluble CRF1 isoforms can have a dual effect on CRF1α activity depending on the intracellular *vs*. extracellular localization.

Key words: *corticotropin-releasing factor, CRF receptor type 1, CRHR1, CRF1, alternative splicing, AtT-20, soluble isoforms.*

## INTRODUCTION

Corticotropin releasing factor receptor type 1 (CRF1) is the major regulatory element of hypothalamus-pituitary-adrenal axis (HPA), which is accountable for global response to the stress (1, 2). In addition, many peripheral organs such as skin, placenta, bowel, colon, kidney, gonads express elements of HPA axis in order to provide locally targeted response to environmental, physiological or pathological conditions (1-5). In mammals, there are two types of CRF receptor (Type 1 and 2) and their expression is tissue dependent and regulated by multiple stress factors (1, 2, 4, 6-9). These two types of CRF receptor differ in affinity to CRF and related peptides (urocortin 1-3; UCN1-3). CRF1 receptor is preferentially activated by CRF and UCN1 and has low affinity for UCN2, which is in contrast to CRF2 (1, 2, 7, 8, 10). In human CRF1 and CRF2 mRNAs undergo alternative splicing generating variety of isoforms with at least 8 found for CRF1 (α, β, c, d, e, f, g and h) and 4 for CRF2 (α, β, γ and soluble α) (1, 2, 7, 11, 12). There is a growing evidence that alternative splicing of CRF receptor mRNA is regulated by many factors including ultraviolet radiation (UVR), cyclic adenosine monophosphate (cAMP), phorbol 12-myristate 13-acetatate (PMA), cell density, onset of labor or pathological conditions (6, 7, 10, 13-15). Moreover, it is possible that, at least partially, alternative splicing of CRF1 receptor may be responsible for changes in responsiveness of HPA system to different stimuli, such as lipopolisacharide, nitric oxide or prostaglandins (1, 4, 5, 7, 14, 16, 17).

Isoforms α and β represent full-length receptor. In contrast to other isoforms, exon 6 is not spliced out of isoform CRF1β mRNA. CRF1 splicing variants c-h, represent different types of deletion/insertion of exons resulting with partial or full exclusion of the substrate binding domain (SBD; isoforms: c, e) or the seven transmembrane domain (7TM; isoforms: d, e, f, g, h). The expression of several CRF1 isoforms was detected in the skin of different species (human, mouse, hamster) and in variety of other organs or tissues, including: kidney, digestive system, myometrium, immune cells, retinal pigment and many others (6, 7, 10, 13, 14, 18-20).

Previously, using two different cellular models (simian kidney - COS-7 and human HaCaT keratinocytes), we have shown that alternatively spliced isoforms of CRF1 receptor play

important function in modulation of CRF1α activity and its cellular localization. Additionally, the expression of different isoforms has an influence on downstream activation of cAMP, inositol triphosphate or calcium signaling (14, 21).

Recently we have postulated that the expression of multiple splicing variants of CRF1 regulate signaling through main isoform, namely - CRF1α (7, 14). Specifically, expression of CRF1 isoforms with impaired C-terminal, 7-TM domain (isoforms: d, f, g) modulate activity of CRF1α by preventing it from proper membrane localization in epidermal keratinocytes. The soluble isoforms of CRF1 (e and h) lacking 7TM domains were also secreted into the media (14). Similar, secretion was observed for construct mNT-CRFR1 (containing amino acids 1-119 of native CRF1) and showed for soluble isoform of CRF2 (sCRF2α) (22, 23). Full SBD seems to be sufficient for at least some of receptor function, as mNT-CRFR1 and sCRF2α were shown to bind CRF analogs (22-25) and modulation of downstream CRF signaling by overexpression of CRF1h was demonstrated (14, 26).

Although expression of CRF1 isoforms in many different cellular systems is already documented (6, 7, 10, 19, 20, 27-29), the significance of alternative splicing of CRF1 receptor is far from being understood. In order to gain more information on this process we have used mouse pituitary cell line AtT-20 as a study model. The library of human CRF1 isoforms tagged with EGFP, dsRFP or without a tag was used to study localization, co-localization and activity of CRF1 splicing variants with special attention to soluble isoforms. The presented data indicates that the expression of human CRF1 isoforms in mouse pituitary cell line AtT-20 can modulate CRF signaling, by altering localization and activity of the main isoform CRF1α. Moreover, such modulation can change dramatically, when soluble isoforms are secreted into the media.

## MATERIALS AND METHODS

### *Cell culture, transfection and luciferase assay*

Mouse pituitary AtT-20 cells were grown in F10 medium supplemented with fetal bovine serum (5%) and 1% penicillin/streptomycin/amphotericin antibiotic solution (Sigma Chemical Co., St. Louis, MO). Cells were grown until they reached 70-80% and then transfected with plasmids DNA, coding CRF1 isoforms tagged on C-terminus with V5 tags, by using lipofectamine and Plus reagent (Invitrogen, Carlsbad, CA). The dual luciferase reporter gene assays (Promega, Madison, WI) were conducted by using pCRE-luc, pAP1-luc and pCARE-luc or pPOMC-luc vectors containing firefly luciferase gene under control of CRE, AP-1, CARE transcription elements or *POMC* promoter, respectively (30). Plasmid phRL-TK (coding *Renilla* luciferase) was used as normalization control of tranfection efficiency (Promega, Madison, WI). All plasmids used in this study were described previously (19, 30). Twenty four hours after transfection cells were incubated with 1 or 100 nM CRF (Sigma Chemical Co., St. Louis, MO) for 1 to 24 hours (as indicated). Luciferase assay were performed at least in triplicates, background was subtracted and values for firefly luciferase were divided by *Renilla* luciferase. The relative transcriptional activity was expressed as a fold change in comparison to untreated control.

### *Visualization of CRF1 isoforms in AtT-20*

AtT-20 cells were seeded in 8 well Lab-Tek II chamber slides (Nalge Nunc, Inc., Naperville, IL). Cells were tranfected with plasmid DNA coding CRF1α isoforms tagged with EGFP or/and dsRFP (only CRF1α) at 70-80% of confluence. After 24-48 hours

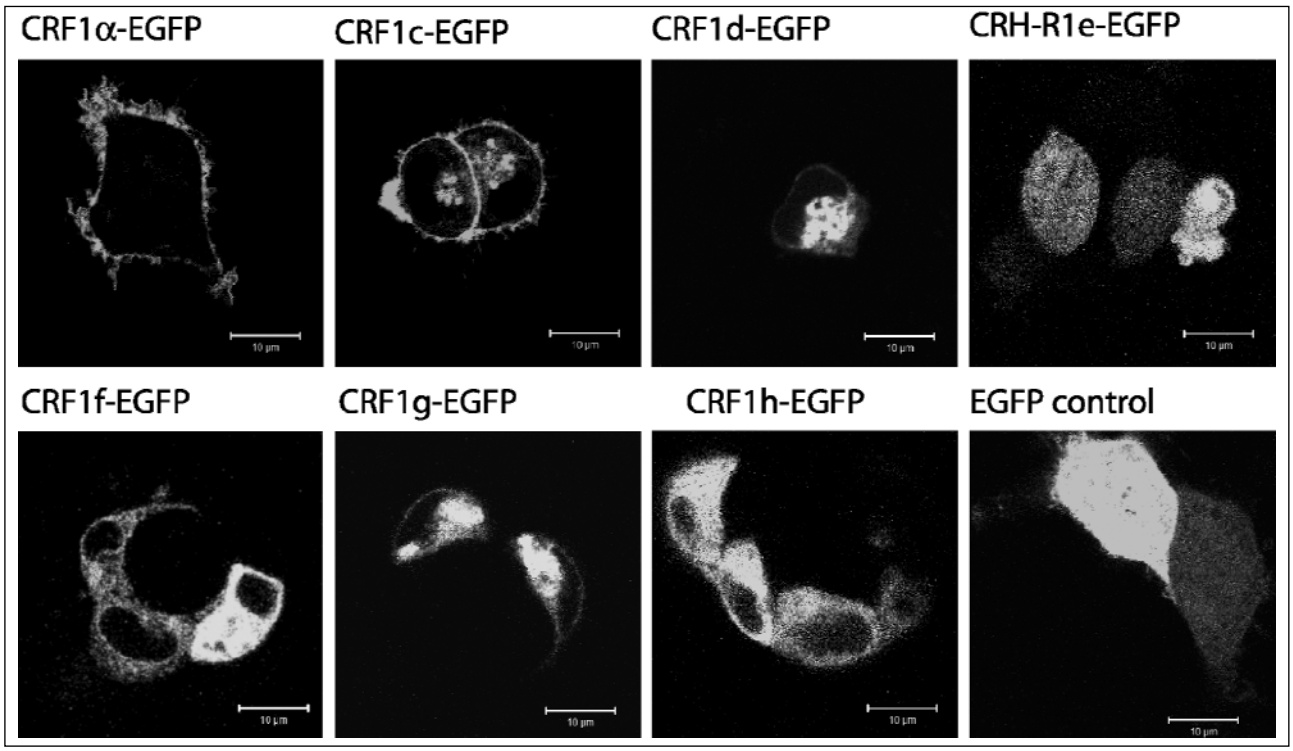


*Fig. 1.* Expression and localization of CRF1-GFP isoforms in AtT-20 cells.
AtT-20 cells were transfected with plasmids containing cDNA of CRF1 isoforms α, c, d, e, f, g or h fused with EGFP on C-terminus. Twenty four hours after transfection cells were fixed and observed under laser scanning confocal fluorescent microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Plan-Neofluor oil immersion 40x objective with suitable filter setup.

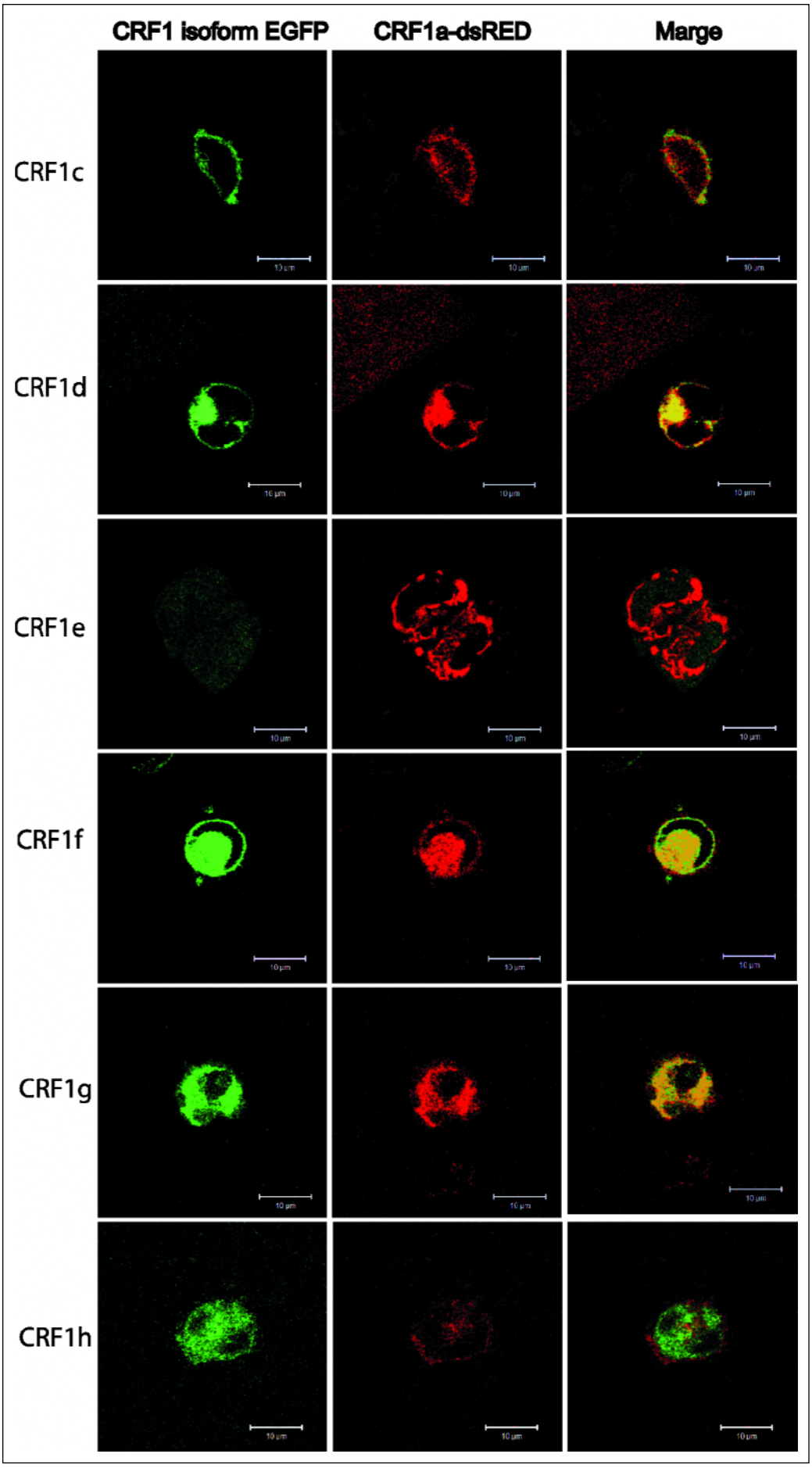


*Fig. 2.* Co-localization of CRF1α-dsRFP with isoforms c, d, e, f, g and h. AtT-20 cells were co-transfected with CRF1-dsRFP (red) together with other CRF1 isoforms tagged with GFP (green). Slides were observed *in vivo* with laser scanning confocal fluorescent microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Plan-Neofluor oil immersion 40x objective with suitable filter setup.

cells were washed, fixed or observed *in vivo* with the laser scanning confocal fluorescent microscope (LSM 510, Carl Zeiss GmbH, Jena, Germany) equipped with Plan-Neofluor oil immersion 40x objective with suitable filter setup. Representative images were acquired showing nuclear cross-sections.

*Statistical analyses*

Data is presented as mean±SEM (n=3–6), and is analyzed with a Student's t-test (for two groups) or one-way analysis of variance with appropriate post-hoc tests (for more than two groups) using Graph Prism 4.00 (GraphPad Software, San Diego, CA). Statistically significant differences are denoted with asterisks: *P<0.05, **P<0.005.

## RESULTS

*Subcellular localization of human CRF1 isoforms expressed in AtT-20 cells*

In order to elucidate intracellular localization of human CRF1 isoforms the library of plasmids expressing CRF1 isoforms α, c, d, e, f, g and h tagged with EGFP was tested on mouse pituitary cell line - AtT-20. As predicted, CRF1α and CRF1c isoforms were found predominantly on the cell membrane (*Fig. 1*). In contrast, isoforms with impaired 7TM domain were retained inside of cells with isoforms d, f and g forming intracellular aggregates (*Fig.1*). Soluble isoform h had similar intracellular localization as d, f and g, but formation of intracellular aggregates was less pronounced. The isoform CRF1e was dispersed uniformly across the cell including nucleus (*Fig.1*).

*Co-expression of alternatively spliced CRF1 isoforms modulates localization of CRF1α*

Co-localization of different isoforms of CRF1 tagged with EGFP and CRF1α fused with dsRFP was studied in AtT-20 cells. As predicted co-expression of isoform α and c resulted in their co-localization in the cell membrane (*Fig. 2*). Isoforms d, f and g also showed co-localization with CRF1α, but mainly in form of intracellular aggregates. Although, soluble isoform CRF1e-EGFP did not co-localize with CRF1α-dsRFP, the CRF1α-dsRFP had a tendency to form aggregates around a nucleus. The CRF1h did not influence proper membrane localization of CRF1α and showed only slight co-localization inside of the cell.

*Modulation of CRF stimulated transcriptional activity in AtT-20 cells by expression or co-expression of CRF1 isoforms*

The effect of expression of human CRF1α in mouse AtT-20 pituitary cells on transcriptional activity of cAMP, $IP_3$ (AP1 promoter) and $Ca^{+2}$ responsive elements (plasmids pCRE-luc, pAP1-luc and pCARE-luc, respectively) was measured by dual luciferase reporter assay. Under experimental conditions, control AtT-20 cells poorly responded to CRF at concentration 1 nM, *e.g.*, only slight stimulation was observed (*Fig. 3*). However, treatment of AtT-20 cells transected with CRF1α, with 1 nM CRF, resulted in time dependent stimulation of CRE, AP1 and CARE responsive elements with the highest activity observed 6 and 24 hours after exposure (*Fig. 3*).

*Fig. 4* shows the CRF effect on AP1 activity in cells transfected with different constructs. While significantly higher CRF induced stimulation was observed in cells overexpressing CRF1α in comparison to control, this effect was not seen in cells overexpressing CRF1 isoforms with predominant intracellular localization (CRF1d, f, g and h) (*Fig. 4*). This suggests that proper membrane localization of CRF1 receptor and presence of full 7TM domain is required for expressing functional receptor activity.

*Soluble isoforms modulate activity of CRF1α*

Previously, it was shown that soluble isoforms of CRF receptors (Type 1 and 2) were able to bind CRF or CRF related ligands and they expressed some activity *in vivo* (14). The CRF and UCN stimulated transcriptional activity of pCRE-luc in AtT-20 cells transfected with CRF1α in a dose dependent manner (*Fig. 5A*). In contrast, this effect was absent or minimal in cells overexpressing CRF1h (*Fig. 5B*). Furthermore, CRF significantly stimulated CARE-luc (*Fig. 5C*), AP1-luc (*Fig. 5D*),

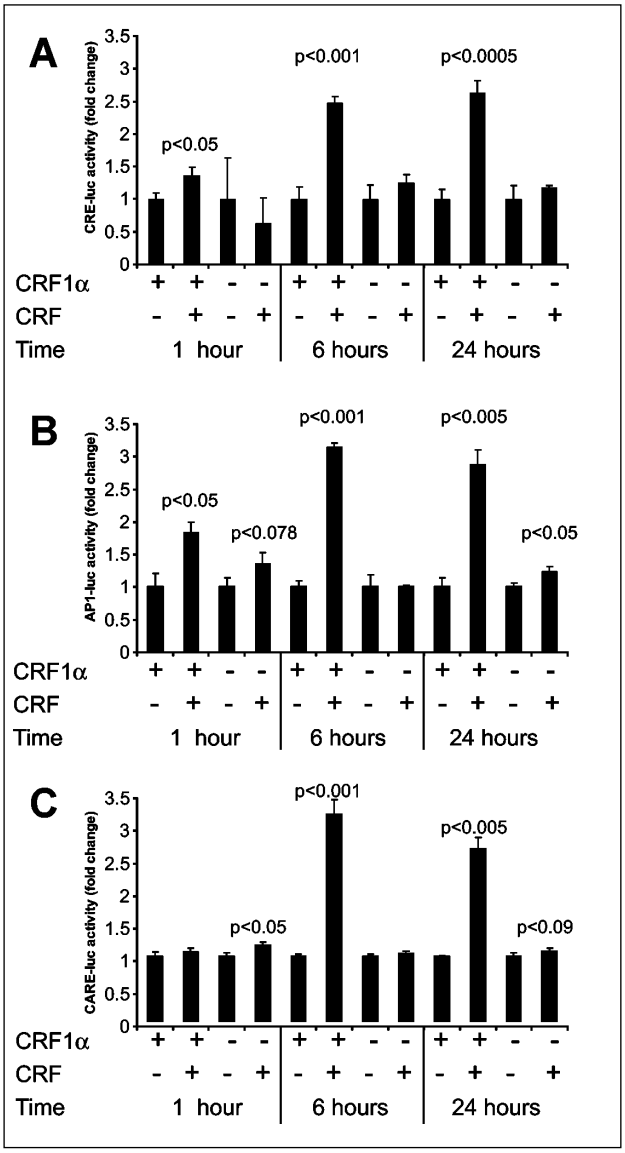


*Fig. 3.* Time dependent stimulation of transcriptional activity of CRE (A), AP1 (B) and CARE elements by CRF (1 nM) in AtT-20 cells transfected with plasmid coding CRF1α isoform. AtT-20 cells were additionally co-transfected with plasmids containing firefly luciferases. Plasmids pCRE-luc AP1-luc or pCARE-luc coded firefly luciferase under control of CRE, AP1 or CARE transcription element, respectively; and plasmid pRL-TK coded *Renilla* luciferase. twenty four hours after transfection cells were treated with CRF (1 nM) for 1, 6 or 24 hours as indicated. The activity of luciferase was measured using a dual luciferase assay. Results were shown as a fold change versus control.

and POMC-luc (*Fig. 5E*) transcriptional activities in cells overexpressing CFR1α, while overexpression of CRF1h had no visible effect (*Fig. 5C-E*).

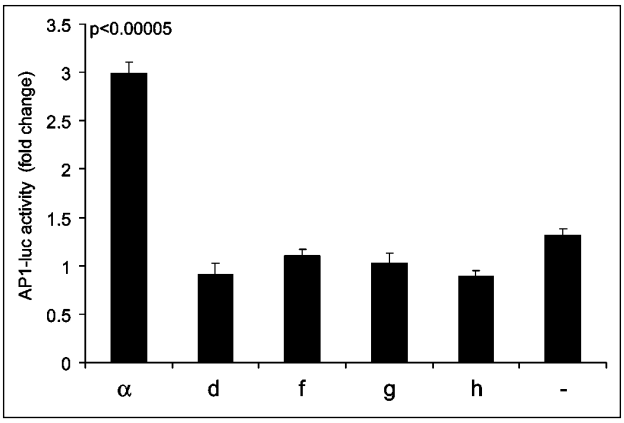


*Fig. 4.* CRF stimulates IP3 responsive element in AtT-20 overexpressing CRF1 isoforms. AtT-20 were transfected with DNA of constructs carrying CRF1 isoforms, AP1-luc (Firefly luciferase under control of AP1 element) and pRL-TK (*Renilla* luciferase). After 24 hours cells were treated with CRF (1 nM) and activity of luciferase was measured after additional 24 hours. Results were shown as a fold change in comparison to control.

In next step, we investigated whether co-expression of CRF1e or h with CRF1α will change its CRF stimulated activity. Surprisingly, overexpression of those isoforms resulted in amplification of CRF induced activity of both CRE (*Fig. 6A*) and AP1 (*Fig. 6C*) responsive elements when compared to the cells overexpressing solely CRF1α. The effect was significantly higher for the CRF1h in comparison to CRF1e (p<0,05).

Since soluble isoforms of CRF receptors (CRF1e, CRF1h and sCRF2) can be released from the cells (14, 22), we tested the effect of conditioned media from AtT-20 cells overexpressing soluble isoforms e and h on CRF1α downstream signaling (*Figs. 6B, D*). Interestingly, media collected from cells expressing CRF1h partially inhibited activation of both CRE and AP1 responsive elements in AtT-20 cells expressing CRF1α and treated with CRF (1 nM). The media from the cells expressing CRF1e had no effect on transcriptional activation of CRE-luc construct (*Fig. 6B*) and weak but statistically significant effect on AP1-luc (*Fig. 6D*). Control media taken from culture expressing CRF1α or empty vector had no effect on transcriptional activation in cells overexpressing CRF1α.

## DISCUSSION

There is growing evidence that alternative splicing of precursor mRNA plays essential role in regulation of transcription and determinate activity of newly synthesized proteins.

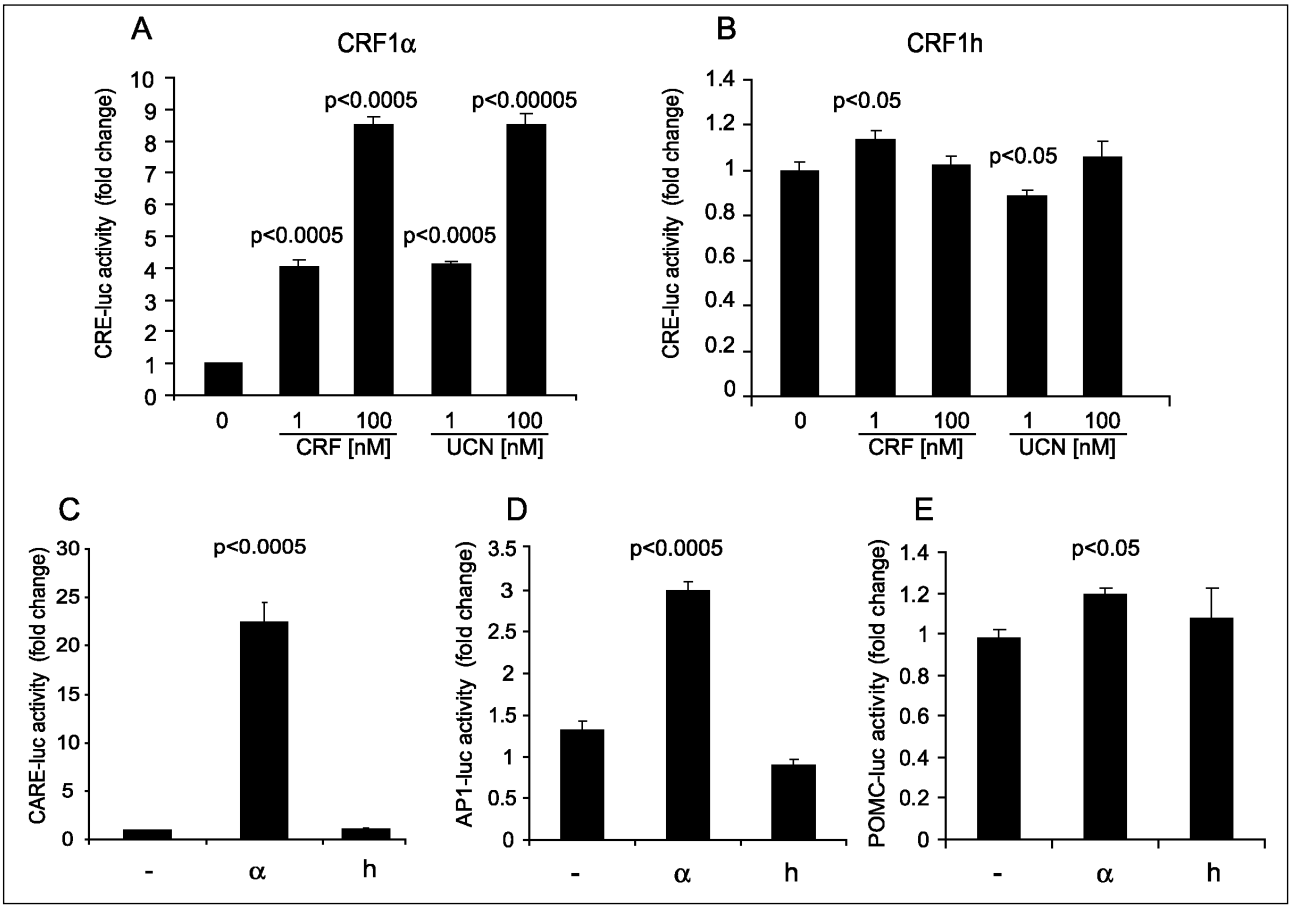


*Fig. 5.* Transcriptional activity of CRE, CARE, AP1 and POMC elements mouse AtT-20 cells expressing CRF1 isoforms α or h. AtT-20 cells were co-transfected with plasmid DNA coding CRF1α (A, C-E) or CRF1h (B, C-E) together with reporter plasmids: pCRE-luc (A,B), pCARE-luc (C), pAP-1-luc (D) or pPOMC-luc (E) and pRL-TK caring *Renilla* luciferase. Cells were treated for 24 hours with CRF or UCN1, both at 1 nM concentration, as indicated (A, B) and activity of luciferase was measured. Results were expressed as a fold change in comparison to control.

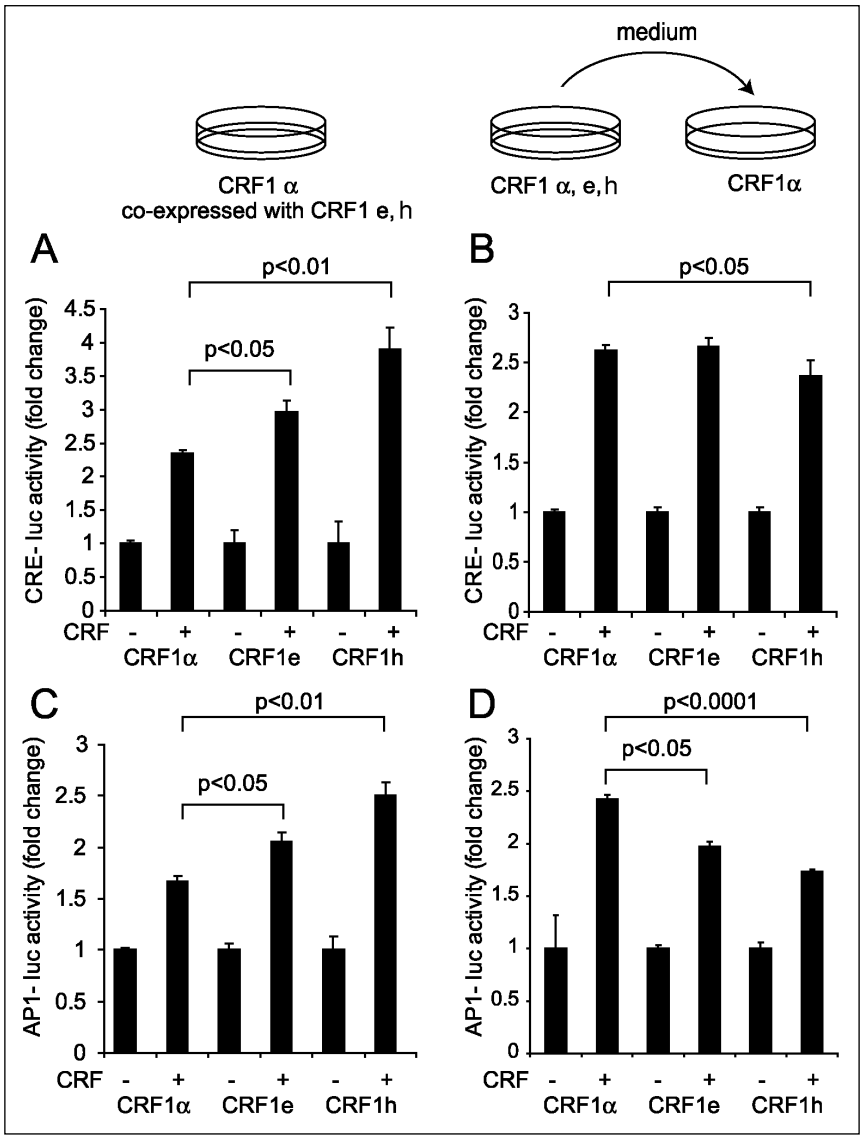


*Fig. 6.* CRF (1 nM) stimulation of CRE (A, B) or AP-1 (C, D) elements in AtT-20 co-expressing CRF1α with CRF1e or h (A, C) or cells overexpressing only CRF1α to which conditioned media from cells overexpressing either CRF1h or CRF1e was added (B, D). AtT-20 cells were co-transfected with DNA of constructs carrying CRF1α, e, h isoforms, pCRE-luc (A, B) or pAP1-luc (C, D), and pRL-TK (*Renilla* luciferase) plasmids and treated with CRF (1 nM) cells 24 hours after transfection. Panels A and C represent co-transfection of CRF1α with soluble isoforms e or h. Panels B and D show effects of conditioned media from cells overexpressing CRF1e or CRF1h on cells overexpressing CRF1α. The activity of luciferase was measured 24 hours after treatment. Results were shown as a fold change in comparison to control.

Nevertheless, the function and significance of alternative splicing among GPCRs still requires clarification. Since it was postulated previously, that expression of alternative (10, 15) spliced isoforms of CRF1 receptor modulates CRF signaling (1, 7, 10, 14, 15) we have examined this phenomenon in more details *(Figs. 1-6)*.

The proper function of the receptor is linked to its membrane localization. As demonstrated on *Fig. 1*, only isoforms α and c showed such localization. Other CRF1 isoforms, with partial or full deletion of 7-TM domains, were found predominantly inside of the AtT-20 cells forming aggregates (isoforms d, f, g and h). Only isoform e was uniformly distributed in cytoplasm and nucleus. This is in an agreement with similar observations made in HaCaT keratinocytes (14) and is further supported by Gramatopoulos group report on partial intracellular localization of CRF1d in HEK-293 cells (31). Interestingly, co-expression of CRF1α with isoforms d, f and g in AtT-20 cells, resulted in co-localization and intracellular aggregation of receptors (*Fig. 2*). Similar effect was observed in HaCaT keratinocytes (14) and it might be explained by interaction of isoforms with formation of oligomers (presumably homo- and heterodimers). Oligomerization is a well-known process of regulation of receptor activity, but until recently was considered as restricted to family A (rhodopsin-like receptors) of GPCRs (32-34). CRF1 (family B1) and its isoforms were found to form high molecular complexes, which were stable during electrophoresis under denaturing conditions (7, 14, 18, 19, 30). In addition, direct formation of CRF1 homodimers (35) or heterodimers with vasopressin V1b receptor was reported recently (36). Thus, oligomerization might explain co-localization and intracellular retention of CRF1α when co-expressing with isoform d, f, and g (*Fig. 2*) (14).

Our current model of CRF signaling through the receptor emphasizes central role of CRF1α and suggests modulatory function of other isoforms with potential formation of oligomers (7, 14). Accordingly, treatment of AtT-20 cells overexpressing CRF1α with CRF resulted in time dependent stimulation of CRE, AP1 and CARE transcription elements and POMC promoter (*Figs. 3* and *5*). In contrary, the expression of CRF1 isoforms: d, e, f, g or h in AtT-20 had minor or no influence on CRF signaling (*Fig. 4*). Therefore, intracellular localization of CRF1d, f and g (*Fig. 1)* (14) can limit the access of ligands to the receptor. The intracellular co-localization of CRF1α with isoforms d, f or g (*Fig. 2* and (14)) might also explain no effect or inhibition of downstream CRF-driven signaling in HaCaT keratinocytes or COS-7 cells (14, 21). Additionally, CRF1d showed limited coupling to G proteins ($G_s$, $G_i$ $G_o$ and $G_q$) and attenuated coupling to adenylate cyclase, in comparison to CRF1α (29). Furthermore, urocortin 2 is known to stimulate cAMP production, ERK1/2 and p38MAPK in HEK-293 cells

overexpressing CRF2β, but this effect is abrogated by co-expression of CRF2β with CRF1d (29, 31). Taken together, these observations indicate that intracellular localization of CRF1 isoforms with impaired 7-TM domain (CRF1 d, f or g) modulate localization of CRF1α through formation of oligomers, resulting in potential inhibition of CRF signaling.

Isoforms CRF1e and h, identified previously as soluble isoforms (7, 10, 20, 21), lack entire 7TM domains that prevents them from proper membrane localization (*Fig. 1*. and *2)* (14). CRF1h is a naturally occurring model of SBD of CRF1, with addition of "cryptic' exon of unknown function (10). Similar construct - mNT-CRFR1, containing only SBD of CRF1, was extensively investigated by Perrin and coworkers and its secretion and ligand binding properties were shown (23, 24). On the other hand, CRF1e posses only 40 amino acids of the native receptor, including 23 amino acids of a signal peptide and 114 amino acids on C-terminus without any homology to know proteins (12, 17). This indicates that CRF1e isoform lacks proper SBD with characteristic disulfide bridges (23, 24), which should impair the ligand binding. In addition, previous demonstration that expression of CRF1e-EGFP in HaCaT keratinocytes was not stable in comparison to other isoforms, suggesting fast turnover of this isoform protein or mRNA and its potential role as a decoy mRNA/protein (7, 22).

We have also shown that soluble isoforms (CRF1e and h), similarly to mNT-CRFR1 (23) and sCRF2α (22), were secreted to the media and this effect was more pronounced for CRF1h (14). Therefore, potential activity of soluble isoforms in the media was compared to the effect of its intracellular expression. CRF1e and h, when expressed alone, had none or minimal effect on CRF signaling (*Figs. 4* and *5*) (14, 21). In contrast, overexpression of isoforms e or h had stimulatory effect on CRF1α-mediated activation of CRE and AP1 transcription elements after CRF treatment (*Figs. 6A, C*). In contrast, use of conditioned media showed that CRF1α-driven stimulation of CRE and/or AP1 transcription elements by CRF was inhibited in the presence of medium collected from cells overexpressing isoforms e or h (*Figs. 6B, D*). Therefore, we postulate that soluble isoforms, when expressed intracellularly, modulate CRF signaling by influencing CRF1α trafficking or recycling. It is also possible that CRF1h, when present at a cell membrane works as an additional "antenna" for CRF1α. It has to be noted that, although soluble isoforms have not co-localized with CRF1α or cell membrane, they form high molecular weight complexes suggesting possible oligomerization (14, 21). While, detailed mechanism of intracellular activity of soluble isoforms requires additional studies, experiments with the conditioned media suggest that CRF1h (and to a lesser extend CRF1e), when secreted to the media, attenuate the activation of membrane bound receptors, either *via* sequestration of the ligand (CRF1h) or *via* another mechanism (CRF1e) that remains to be defined. This decoy soluble receptors activity may explain partial inhibition of downstream CRF1α signaling as shown in *Fig 6* and previously (14, 21).

In conclusion, the presented results not only support the central role of CRF1α in CRF signaling but also provide an evidence for modulatory functions of other isoforms with emerging role of soluble isoforms as dual intra- and extracellular modifiers with opposite effects depending on the localization.

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Conflict of interests: None declared.

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