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Role of cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) in regulating the trafficking and signaling of corticotropin-releasing factor receptor 1

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ABSTRACT

Corticotropin releasing factor (CRF) receptor1 (CRFR1) is associated with psychiatric illness and is a proposed target for the treatment of anxiety and depression. Like many G protein-coupled receptors (GPCRs), CRFR1 harbors a PDZ (PSD95/Disc Large/Zona Occludens 1)-binding motif at the end of its carboxyl terminal tail. The interactions of PDZ proteins with GPCRs are crucial for the regulation of their receptor function. In the present study, we characterize the interaction of the cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) with CRFR1. We show using co-immunoprecipitation that the two proteins interact in human embryonic kidney (HEK293) cells in a PDZ motif-dependent manner. We find that the interaction occurs at the Golgi apparatus and that overexpression of CAL retains a proportion of CRFR1 in the intracellular compartment and reduces trafficking to the cell surface. We also demonstrate a significant reduction in the levels of receptor at the plasma membrane upon CAL overexpression, as well as a reduction in internalization. We find that the overexpression of CAL in HEK293 cells resulted in a significant reduction in CRF-stimulated extracellular-regulated protein kinase 1/2 (ERK1/2) phosphorylation, but has no effect on cAMP signaling mediated by the receptor. This effect was dependent on an intact PDZ motif and knockdown of CAL expression using CAL siRNA results in a significant enhancement in ERK1/2 signaling. We show that CAL contributes to the regulation of CRFR1 glycosylation and utilize glycosylation-deficient CRFR1 mutants to further examine the role of glycosylation in the cell surface trafficking of CRFR1. We find that the mutation of Asn residues 90 and 98 results in a reduction in cell surface CRFR1 that is comparable to the effect of CAL overexpression and that these mutants are retained in the Golgi apparatus. Mutation of Asn residues 90 and 98 also results in a reduction in the efficacy for CRF-stimulated cAMP formation mediated by CRFR1. Taken together, our data suggest that CAL can regulate the anterograde trafficking, the internalization as well as the signaling of CRFR1 via modulating the post-translational modifications that the receptor undergoes at the Golgi apparatus.

1. Introduction

Corticotropin-releasing factor (CRF) is a 41 amino acid polypeptide commonly secreted from the paraventricular nucleus of the hypothalamus in response to stress subsequently leading to the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) [1,2]. CRF activates two Class B G protein-coupled receptor (GPCR) subtypes, CRFR1 and CRFR2, which are widely expressed in the brain (neocortex and cerebellum) and pituitary [3,4]. CRF can regulate the endocrine response to stress by triggering the anterior pituitary gland to release adrenocorticotropin hormone (ACTH) into circulation. ACTH then stimulates the secretion of glucocorticoids from the adrenal cortex. Glucocorticoids regulate various functions both in the central nervous system (CNS) and at the periphery [5]. In addition, studies have shown a link between pathophysiological changes in the CRF system and various neuropsychiatric disorders such as major depression, panic disorder, anorexia

Abbreviations: $5HT_{2A}R$, Serotonin 2A receptor; ACTH, Adrenocorticotropin hormone; ANOVA, Analysis of variance; CAL, CFTR-associated ligand; cAMP, Cyclic adenosine monophosphate; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; CNS, Central nervous system; CRF, Corticotropin-releasing factor; CRFR1, CRF receptor 1; CRFR2, CRF receptor 2; EPAC, Exchange proteins directly activated by cAMP biosensor; ERK1/2, Extracellular signal-regulated kinase; FIG, Fused in glioblastoma; GASPs, GPCR-associated sorting proteins; GOPC, Golgi-associated PDZ and coiled-coil motif-containing protein; GPCR, G protein-coupled receptor; HA, Hemagglutinin; HEK 293, Human embryonic kidney 293; HPA axis, Hypothalamic-pituitary-adrenal axis; MAGI, Membrane-associated guanylate kinase inverted; MAPK, Mitogen-activated protein kinase; mGluR, Metabotropic glutamate receptor; PDZ, PSD95/Disc Large/Zona Occluden; PIST, PDZ protein interacting specifically with TC10; SAP97, Synapse-associated protein 97; SSTR, Somatostatin receptor; TGN, Transgolgi network; β_1AR, β_1-adrenergic receptor.

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nervosa, and Alzheimer's disease [5,6]. CRFRs are primarily coupled to $G\alpha_s$ for the activation of adenylyl cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) [7] as well as the mitogen-activated protein kinase (MAPK) signaling pathway [8].

GPCRs can interact with a wide range of different proteins that control the trafficking, cell surface expression and signal transduction. Examples of these proteins include: β-arrestins, GPCR-associated sorting proteins (GASPs), small GTPases and PSD95/Disc Large/Zona Occludens (PDZ) proteins [9]. Many GPCRs, including CRFR1, encode a short class 1 PDZ domain recognition motif (S/T-x-ϕ, where ϕ is any aliphatic amino acid residue) at the end of the carboxyl-terminal tail. PDZ proteins are comprised of one or more 90 amino acid residue protein interaction domains that are thought to scaffold signaling complexes [10,11]. PDZ proteins function to regulate the subcellular localization, trafficking and signal transduction of multiple GPCRs and different PDZ proteins have both overlapping and distinct roles in the regulation of GPCR activity [9–12].

It is now recognized that PDZ proteins have distinct functions depending upon the particular GPCR with which they are associated. For example, MUPP1 interactions enhance $GABA_B$ receptor signaling [13], whereas MUPP1 functions to uncouple the melatonin-1 receptor from $G\alpha_i$ [14] and facilitates resensitization of the $5HT_{2C}R$ [15]. In the case of the β_1-adrenergic receptor (β_1AR), MAGI-2 has been shown to promote β_1AR internalization, whereas MAGI-3 selectively attenuates β_1AR-mediated ERK1/2 signaling [16,17]. In contrast, PSD95 inhibits the internalization of β_1AR and facilitates the formation of β_1AR heterodimer with N-methyl-D-aspartate receptors [18]. SAP97 functions to antagonize the internalization of both CRFR1 and $5HT_{2A}R$, whereas PDZK1 reduces the extent of $5HT_{2A}R$ internalization, but has no significant effect on the endocytosis of CRFR1 [19–21]. Although SAP97 regulates GPCR ERK1/2 signaling in a receptor-interactionindependent manner, PDZK1 overexpression increases CRFR1- but not $5HT_{2A}R$-mediated ERK1/2 activity.

The Golgi-localized cystic fibrosis transmembrane conductance regulator-associated ligand (CAL) is a single PDZ-domain containing protein with two coiled-coil motifs and two evolutionally conserved regions which are required for its Golgi localization [22–24]. CAL is also known as GOPC, PIST and FIG. CAL overexpression reduces the cell surface expression of the β_1AR and mGluR1a by retaining the receptor inside the cell via a direct interaction [25,26]. CAL interacts with mGluR5a and stabilizes receptor expression by a mechanism that is proposed to involve the inhibition of ubiquitination-dependent degradation [27]. CAL also contributes to the regulation of somatostatin receptor recycling and degradation [28,29].

In the present study, we have identified CAL as a protein that interacts with CRFR1. We find that CAL overexpression leads to the retention of CRFR1 in the Golgi and alters the post-translational N-glycosylation status of the receptor, suggesting that CAL negatively regulates the anterograde transport of CRFR1 from the ER-Golgi to the plasma membrane surface. We also find that CAL knockdown enhances ERK1/2 phosphorylation in response to CRFR1 activation, but that CAL expression does not influence cAMP signaling. Taken together, our results indicate that CAL interactions with CRFR1 play an important role in regulating the maturation and cell surface expression of CRFR1.

2. Materials and methods

2.1. Materials

G protein beads were purchased from GE Healthcare (Oakville, ON, Canada). CRF was purchased from R&D Systems (Minneapolis, MN, USA). HA peroxidase high affinity antibody was purchased from Roche (Mississauga, ON, Canada). Rabbit anti-GFP antibody was obtained from Invitrogen/Life Technologies (Burlington, ON, Canada). ECL Western blotting detection reagents were purchased from Bio-Rad (Mississauga, ON, Canada). Alexa Fluor 647 anti-mouse IgG antibody was purchased from Invitrogen/Molecular Probes (Burlington, ON, Canada). Rabbit CAL antibody was purchased from Abcam (Toronto, ON, Canada). Rabbit anti-phospho-p44/42 MAP kinase (Thr202/Tyr402), and rabbit anti-p44/42 MAP kinase antibodies were obtained from Cell Signaling Technology (Pickering, ON, Canada). Mouse anti-HA antibody, endoglycosidases F and H and all other biochemical reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Plasmids

HA-CRFR1 and HA-CRFR1 ΔTAV were described previously [30,31]. Human CAL was kindly provided by Dr. Randy Hall (Emory University School of Medicine). The CAL cDNA was subcloned into the expression vector pEYFP-N1. CAL siRNA (Hs_GOPC_3) GCUGCAGCUUCAUGCUAA ATT was purchased from Qiagen (Toronto, ON, Canada). For the negative controls, we used Silencer Negative Control #1 AM4635 AGUACU GCUUACGAUACGGTT from Invitrogen (Burlington, ON, Canada). The exchange proteins directly activated by cAMP biosensor (EPAC) was a gift from Drs. Ali Salahpour (University of Toronto) and Marc Caron (Duke University) [32]. TGN-CFP was kindly provided by Dr. Stephen Pasternak (University of Western Ontario). Site-directed mutagenesis was used to introduce N to Q in the following constructs: CRFR1 N38Q, CRFR1 N78Q, CRFR1 N90Q and CRFR1 N98Q and mutations were confirmed by sequencing.

2.3. Cell culture and transfection

Human embryonic kidney (HEK 293) cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum. Cells were plated on 10-cm dishes 24 h prior to transfection. All experiments were performed on 75–80% confluent plates. Transfections were performed using calcium phosphate protocol except for siRNA transfections which were performed using Lipofictamine 2000 following manufacturer's instructions. Transfections were performed with 1 μg of each construct. Empty pcDNA3.1 vector was used to equalize the total amount of plasmid cDNA used to transfect cells. 18 h post-transfection, cells were washed with phosphate-buffered saline (PBS) and resuspended with trypsin, 0.25% EDTA. Experiments were performed approximately 48 h after transfection except for knockdown experiments which were performed 72 h post transfection since this protocol resulted in the maximum knockdown of CAL.

2.4. Co-immunoprecipitation

24 h after transfection, HEK 293 cells were seeded onto 10-cm dishes. Cells were starved with HBSS for 1 h at 37 °C then stimulated with 100 nM CRF agonist for 30 min. Cells were then lysed in 500 μL lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100) containing protease inhibitors (1 mM AEBSF, 10 μg/ml leupeptin, and 2.5 μg/ml aprotinin) for 20 min on a rocking platform at 4 °C. Samples were collected into 1.5 ml Eppendorf tubes and centrifuged at 15,000 g for 15 min at 4 °C to pellet insoluble material. A Bradford protein assay was performed, and 150 μg of protein was incubated for 2–4 h at 4 °C with protein G-Sepharose and mouse anti-HA antibody (1:50). Beads were washed three times with cold lysis buffer and incubated overnight at room temperature in 3× SDS loading buffer containing 2-mercaptoethanol. Samples were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and western blots were then performed with the indicated antibodies (rabbit anti-GFP, 1:10000), (HA-POD, 1:1000).

2.5. Confocal microscopy

HEK 293 cells were co-transfected with HA-CRFR1, CAL-YFP and TGN-CFP in 10-cm dishes and 24 h after transfection, cells were reseeded onto glass coverslips. 24 h later, cells were washed with

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