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Identification and characterization of two novel (neuro)endocrine long coiled-coil proteins

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Abstract **We have identified a novel vertebrate-specific gene by applying a Differential Display method on two distinct subtypes of pituitary melanotropes showing divergent secretory phenotypes of hypo- and hypersecretion. A paralogue of this gene was also identified. The existence of a long coiled-coil domain and a C-terminal transmembrane domain in the sequences, together with the Golgi distribution of the proteins in transfected cells, suggest that they can be considered as new members of the golgin family of proteins. Both genes were primarily expressed in (neuro)endocrine tissues in vertebrates thus supporting a role for these proteins in the regulated secretory pathway.**

Keywords: Secretory pathway; (Neuro)endocrine cells; Cloning; Gene expression; Long coiled-coil proteins; Golgins

1. Introduction

The regulated secretory pathway is an intricate, multi-step process that involves generation of transport carriers, sorting and packaging of specific cargo proteins, delivery of transport carriers to the plasma membrane, and membrane fusion in a regulated manner [1]. (Neuro)endocrine cells tightly control particular stages of this process by synthesizing a wide variety of regulatory proteins with specific functions within the regulated secretory pathway. Many of these proteins have been already identified; for instance, protein coats (i.e. COPI and COPII export machineries) mediating vesicle budding and cargo selection [2], the family of small GTPase Rab proteins which play specific regulatory roles in controlling intracellular vesicle traffic [3], tethering factors such as Golgins [4], or the family of SNARE proteins, which control vesicle targeting, docking and fusion [5]. However, a considerable effort is still being devoted to isolate and characterize new proteins involved in the secretory pathway.

In this scenario, α-melanocyte stimulating hormone (α-MSH)-producing melanotropes of the intermediate lobe of the amphibian pituitary comprise a valuable model to identify and characterize new proteins involved in the regulated secretory pathway, as this cell type is composed of two distinct melanotrope subpopulations exhibiting opposite secretory phenotypes, which can be easily separated by means of classical cell separation techniques (i.e. centrifugation in Percoll density gradients). More specifically, one subpopulation comprises melanotropes showing low intracellular α-MSH content but high spontaneous secretory activity, and a strong secretory response to stimulatory secretagogues, and can thus be defined as highly active secretory cells. In contrast, the other subpopulation is composed of melanotropes featuring a hormone-storage phenotype, as they display high amount of α-MSH-containing secretory granules but low secretory activity, both under basal conditions and in response to stimulatory factors. In amphibians, in which α-MSH regulates skin pigmentation in response to background colour conditions, the existence of melanotrope subpopulations enables to finely regulate the amount of hormone produced by the intermediate lobe, so that secretory melanotropes predominate when a high amount of α-MSH is required (i.e. in animals adapted to black background colour conditions) whereas storage melanotropes are more abundant under conditions of low hormonal demand (i.e. in white background-adapted animals) [6,7]. In accordance with their distinct secretory activity, the two melanotrope subpopulations also differ in the expression of key components regulating the processing and intracellular transport of α-MSH such as prohormone convertases and granins [8,9].

Armed with this cellular model, in this work we have applied a Differential Display (DD) methodology to compare the genetic fingerprint of secretory and storage melanotropes. Among the isolated sequences, we identified a new gene of unknown function, which is preferentially expressed in the storage cell subset. We report herein the molecular characterization of this gene as well as its tissue and subcellular distribution. Additionally, we have also identified and characterized a paralogue sequence of this gene.

2. Materials and methods

2.1. Animals

Adult frogs (*Rana ridibunda*) (Ranas Orense, Orense, Spain) were maintained at 8 °C on a 12 h (light/dark) photoperiod. Adult Wistar rats were maintained under constant conditions of light and temperature (14 h of light at 22 °C), and fed ad libitum. The animals were killed by decapitation, and tissues were dissected and stored at −80 °C until use. Animal care and experimental procedures were approved by the Bioethics Committee of the University of Cordoba.

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2.2. Cell dispersion and isolation of melanotrope subtypes

Neurointermediate lobes (NIL) of the frog pituitary were transferred to sterile Leibovitz culture medium (L-15, Sigma Chemical, London, UK) and supplemented with 1 mM glucose and 0.4 mM $CaCl_2$ (pH 7.4). NILs were enzymatically and mechanically dispersed as described previously [10]. For separation of the two melanotrope subsets, dispersed cells ($1–2 \times 10^6$ cells in 250 μl of culture medium) were layered on a 9-ml hyperbolic density gradient (1.027–1.072 g/ml) of Percoll (Pharmacia LKB, Uppsala, Sweden). After gradient centrifugation ($3000 \times g$, 25 min, 4 °C), fractions containing storage and secretory melanotropes (fractions 1 and 5–7, respectively) were collected, washed in L-15 and stored at −80 °C until use.

2.3. RNA isolation

Total RNA from whole frog neurointermediate lobe or from the separate melanotrope subsets was isolated using the RNA-easy Mini Kit columns (Qiagen, Hilden, Germany) followed by on column DNase RNase-free treatment (Qiagen). Total RNA from rat and frog tissues was isolated using TRIzol reagent (Invitrogen, Barcelona, Spain) according to the manufacturer's protocol. A panel of total RNA from human tissues was purchased from BD Biosciences Clontech (Erembodegen, Belgium).

2.4. Differential display reverse transcriptase-polymerase chain reaction

DDRT-PCR was performed on DNase-treated total RNA from melanotrope subpopulations using the HieroglyphTM mRNA Profile Kit (Genomyx, Beckman Instruments, Fullerton, CA). The RNAs (0.2 μg) were converted to cDNA by reverse transcription using 4 pmol of each anchor primer. cDNA synthesized from 10 ng of total RNA was amplified with a combination of arbitrary primers and ^{32}P-labeled anchor primers. The cycling parameters were as follows: a denaturation step of 95 °C for 2 min, followed by 4 cycles of 92 °C for 15 s, 46 °C for 30 s, 72 °C for 2 min and they were followed by 25 cycles of 92 °C for 15 s, 60 °C for 30 s, and 72 °C for 2 min. Amplified cDNA was then subjected to electrophoresis on 6% denaturing polyacrylamide gel and visualized by autoradiography. The cDNA bands preferentially expressed in either secretory or storage melanotropes were reamplified by PCR, cloned into pGEM-T vector (pGEM-T Vector System I, Promega Corp., Madison, WI) and sequenced (Central Sequencing Service, University of Cordoba, Spain).

2.5. Molecular cloning of frog KIAA0555 cDNA

Among the sequences obtained by DDRT-PCR, we found and selected for further studies a nucleotide fragment preferentially expressed in the storage melanotrope subset which showed high identity with a human cDNA named as KIAA0555. The cDNA fragment isolated by DD was used as template to label a 430-bp sequence using the DIG Labeling Mix Kit (Roche, Mannheim, Germany) and the forward and reverse primers (5′-ATT CGT AGA CTG ATG GAT-3′ and 5′-CCA TAC GTT GGA AGG ATT-3′). This digoxigenin-labeled fragment was used as a probe to screen a pituitary cDNA library from *R. ridibunda* [11]. This enabled the identification of a single positive clone which was subsequently isolated and sequenced.

2.6. Expression analysis by RT-PCR

Total RNA from different tissues and species (2 μg), primed with random primers, was reverse-transcribed into first-strand cDNA at 42 °C for 1 h using the PowerScript™ Reverse Transcriptase (BD Biosciences Clontech) in a 20-μl volume, and stored at −20 °C until use. PCR was performed in a 25-μl mixture containing 100 ng template cDNA, 20 pmol of each primer, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 2 U Taq DNA polymerase (Ecogen, Barcelona, Spain). The RT-PCR primers used are the following: forward frog KIAA0555, 5′-CCA GGC ATA CTT GCG AA-3′; reverse frog KIAA0555, 5′-AAG GGC TCA TCC GTG TT-3′; forward rat KIAA0555, 5′-TGA GGA CGG CTG TAG AA-3′; reverse rat KIAA0555, 5′-TGC CAT CTT TGA AGG TTT A-3′; forward human KIAA0555, 5′-TGA GGA CGG CAG TAG AA-3′; reverse human KIAA0555, 5′-GCC ATC TTT GAA GGT TTA G-3′; forward rat KIAA4091, 5′-CCT GAA CAG CAG TTG GAC GA-3′; reverse rat KIAA4091, 5′-CTT CTC TCA GCC GCT TTA GCA G-3′; forward human KIAA4091, 5′-GCG GGA CAA GCT GTT AAG ATT C-3′; reverse human KIAA4091, 5′- AAG CCT CTT CGT CGT ATC CAA A-3′; forward GAPDH, 5′-TTT CAC CGC TAC ACA GAA G-3′; reverse GAPDH, 5′-GTT GCT GTA ACC GAA TTC A-3′; forward HPRT, 5′-CAG TCC CAG CGT CGT GAT TA-3′; and reverse HPRT, 5′-AGC AAG TCT TTC AGT CCT GTC-3′. In addition, specific forward and reverse primers were used to confirm the differential expression of KIAA0555 in frog melanotrope cell subtypes (5′-ATA ATC GCC TTC AAC AA-3′ and 5′-AGG ATC TGA CAG TCA AA-3′). Amplification of frog glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and rat and human hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as an internal control. PCR consisted of a first denaturing cycle at 94 °C for 4 min, followed by the number of cycles determined for the optimal amplification of each gene. Each cycle was composed of 30 s at 94 °C for denaturation, annealing for 30 s and extension at 72 °C for 30 s. A final extension cycle at 72 °C for 7 min was included. PCR conditions were chosen to be at the linear phase of amplification to assess a semiquantitative analysis. PCR products were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. Identities of amplicons were confirmed by sequencing.

2.7. Computational analysis

Database searches were performed using BLAST at NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and at Ensembl Genome Browser (http://www.ensembl.org). Sequence alignments and identity percents were obtained using the CLUSTALW algorithm included in the BioEdit Sequence Aligment Editor 5.0.9 software package. Structural analysis of amino acid sequences was carried out using the algorithms included in the Expasy server (http://www.expasy.net/).

2.8. DNA constructs

To characterize the subcellular distribution of the different isoforms of KIAA0555 and its paralogue (KIAA4091), tagged expression vectors were generated for each cDNA. The CDS of the long and short isoforms of human KIAA0555 (GenBank accession nos. EF512550 and BC017354, respectively) were amplified by RT-PCR using cDNA from human source as template and a high fidelity Pfu polymerase (Pfu-Ultra; Stratagene, La Jolla, CA). The mouse KIAA4091 cDNA (GenBank accession number AK220482) was obtained from Kazusa DNA Research Institute (Japan). Coding sequences were cloned in frame to the C-terminus end of the green fluorescence protein (GFP) in the phrGFP-N1 vector (Stratagene) or in frame to the C-terminus end of the cMyc epitope tag in the pCMV-Myc vector (Clontech). In these constructs, GFP or cMyc epitope tag were linked to the corresponding proteins by the amino acid sequences ELSGLRSRAEASNSAVDT or LSAMEARIRST, respectively. A vector coding for the ts045 variant of the vesicular stomatitis virus glycoprotein tagged to GFP (VSVG-GFP ts045; kindly provided by Dr. K. Simons, University of Dresden, Germany) was used for co-localization studies.

2.9. Transient transfections and confocal fluorescence microscopy

HEK293 AD cells were transfected with the expression vectors using Lipofectamine 2000 (Invitrogen), cultured for 24 h and then fixed in 4% paraformaldehyde for 15 min. Cells were examined under a TCS-SP2-AOBS confocal laser scanning microscope (Leica Corp, Heidelberg, Germany). Depending on the cell depth, 5–10z planes were collected and projected in a single image.

Cells transfected with the GFP-tagged constructs were immunostained for the Golgi marker GM130 using a mouse monoclonal anti-GM130 antibody (BD Transduction Laboratories, Lexington, KY) and an Alexa594-conjugated anti-mouse secondary antibody (Invitrogen). Additionally, cMyc-tagged expression vectors of the distinct proteins were used for co-transfection experiments of HEK293 AD cells with the VSVG-GFP ts045 vector. After overnight transfection, cells were incubated at 40 °C for 16 h and either directly fixed or incubated for a further 30 min period at 32 °C before of paraformaldehyde fixation. cMyc fusion proteins were detected by immunofluorescence using a mouse monoclonal antibody against the cMyc epitope tag (Clontech) and an Alexa594-conjugated anti-mouse secondary antibody.

3. Results

3.1. Identification of KIAA0555, a novel vertebrate-specific gene

Among the cDNA sequences identified by DDRT-PCR, we focused on a nucleotide fragment preferentially expressed in