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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

the storage melanotrope subtype, which showed high identity with a human cDNA of unknown function referred to as KIAA0555 (GenBank Accession number AB011127). Using a frog pituitary cDNA library, we isolated a single clone with an insert of 3240 bp containing a predicted coding sequence of 2460 bp and a 3′-untranslated region (3′-UTR) of 479 bp, including a poly-adenylation signal and the corresponding poly-A tail (GenBank Accession number EF512549). An in-frame stop codon (TGA, −129 position) upstream to the start codon (ATG, +1) indicated that the isolated CDS is complete. The predicted ORF encodes a protein of 820 amino acids (Fig. 1A) with an expected molecular weight of 96.5 kDa and a pI of 5.87. Cloning of the frog full-length cDNA allowed designing appropriate RT-PCR primers to confirm the preferential expression of frog KIAA0555 in storage melanotropes (Fig. 1B).

A BLAST search in the gene databases using the isolated nucleotide sequence unveiled the existence of orthologue genes

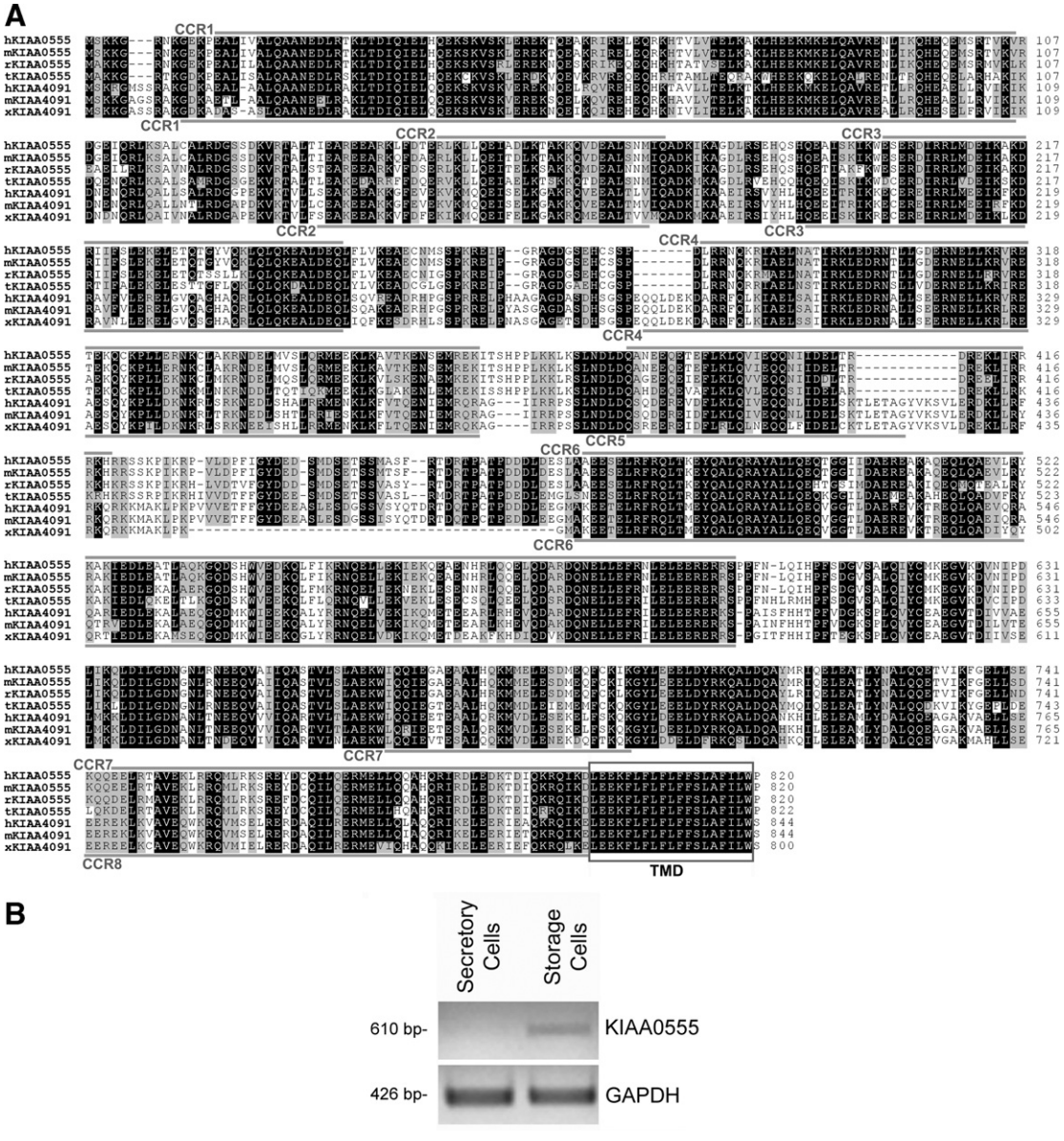

Fig. 1. Comparative analysis of KIAA0555 and KIAA4091 amino acid sequences and expression of KIAA0555 in frog melanotrope subpopulations. (A) Alignment of the complete deduced amino acid sequence for human KIAA0555 (predicted from Ensembl Gene Identification number ENSG00000176049, v43), mouse KIAA0555 (GenBank Accession number XM989629), *Rana ridibunda* KIAA0555 (GenBank Accesion number EF512549), *Takifugu rubripes* KIAA0555 (predicted from Ensembl *T. rubripes* scaffold no. 82, nucleotides from 158000 to 175000, v43), human KIAA4091 (Ensembl Transcript Identification number ENST00000298622, v43), mouse KIAA4091 (GenBank Accession number AK220482) and *Xenopus tropicalis* KIAA4091 cDNAs (predicted from Ensembl *X. tropicalis* scaffold No. 32, nucleotides from 250000 to 315000, v43). Residues in the sequences that are conserved and similar are shown in a black background and a shaded background, respectively. The predicted coiled-coil regions for KIAA0555 are overlined and labeled as CCR1–CCR7. The predicted coiled-coil regions for KIAA4091 are underlined and labeled as CCR1-CCR8. The putative transmembrane domain (TMD) is boxed. (B) For the analysis of the expression of frog KIAA0555 gene in melanotrope subpopulations, total RNA from both secretory and storage melanotropes was isolated and KIAA0555 transcript levels were determined by semiquantitative RT-PCR. GAPDH amplification was used as internal control. The size of each product is shown.

in all vertebrate species already sequenced, but not in non-vertebrate species. Specifically, the homologous human gene (Ensembl Gene Identification number ENSG00000176049, v43) contains 22 exons and 21 introns extending 194 kbp at the chromosomal position 5q32. All introns contain universal donor–acceptor splice sites.

Based on frog KIAA0555 cDNA, we determined the full-length open reading frame of the human, mouse and *Takifugu rubripes* cDNA sequences by database searching. Predicted mammalian and *Takifugu* proteins consisted of 820 and 822 amino acids, respectively. Sequence alignment revealed that the human KIAA0555 protein showed 99%, 88% and 79% identity with the mouse, frog and *Takifugu* KIAA0555 proteins, respectively (Fig. 1A).

3.2. Structural analysis of KIAA0555 protein

Next, we analyzed the structural domains within the amino acid sequence of the human KIAA0555 protein. The Coils algorithm [12] predicted the existence of 7 α-helical stretches of variable length displaying high probability to form coiled-coil structures. These regions span almost the entire molecule in all species analyzed (Fig. 1A). Hydropathy analysis of the predicted KIAA0555 protein revealed the presence of a single hydrophobic region of 19 amino acids at its C-terminus (Fig. 1A, residues 801–819 of human KIAA0555). Use of the HMMTop algorithm [13] revealed that this hydrophobic region likely corresponds to a transmembrane domain with the N-terminal bulk oriented towards the cytosol and, consequently, that the predicted KIAA0555 protein could be considered as a type II transmembrane protein. No signal peptide sequence was detected in the KIAA0555 amino acid sequence.

3.3. Identification of an alternative splicing site for the KIAA0555 gene

A BLAST search in the GenBank database using the full-length ORF predicted for the human KIAA0555 gene (GenBank Accession number EF512550) revealed the existence of a second human cDNA for this gene (GenBank Accession number BC017354). Analysis of the human genomic sequence of KIAA0555 suggested that both cDNAs can be originated by alternative splicing of exon 20, which codes for most of the putative transmembrane domain (residues 805–820). Accordingly, splicing of this exon could generate two isoforms, a long one coding for a protein containing the TMD or a short isoform coding for a protein of 799 amino acids, lacking this domain, and bearing an alternative C-terminus, as the stop codon for the original ORF is also in exon 20 (Fig. 2). Comparative analysis of the human, rat, mouse and *Takifugu* genomes showed that the exon/intron organization of KIAA0555 gene is conserved in these organisms and, therefore, that such alternative splicing event could occur in these species.

3.4. Identification and characterization of a paralogue of KIAA0555

In addition to the orthologue sequences, BLAST search revealed the existence of two KIAA0555-related genes, which were also found exclusively on vertebrate genomes. The first related gene codes for an 844 amino acid protein of unknown function. Interestingly, a mouse full-length cDNA coding for this protein (mKIAA4091, GenBank Accession number AK220482) was isolated from mouse brain in the course of a cDNA sequencing project. Alignment of mouse KIAA0555 and KIAA4091 predicted amino acid sequences revealed a 62% identity. Use of the mKIAA4091 coding sequence to annotate several vertebrate genomes revealed that human KIAA4091 gene (Ensembl Gene Identification number ENSG00000188385, v43) contains 21 coding exons and 20 introns extending 64 kbp at the chromosomal position 10q26. The predicted human protein showed 94% identity with the mouse protein and 84% with the *Xenopus* protein (Fig. 1A).

Structural analysis of human KIAA4091 showed that the predicted protein contains eight regions with a putative coiled-coil structure (Fig. 1A) which, similar to its paralogue KIAA0555, would span almost the entire KIAA4091 sequence. Actually, some coiled-coil regions are highly similar between both paralogues. Additionally, KIAA4091 contains a 19-amino acid hydrophobic region at its C-terminus identical to that of KIAA0555 (Fig. 1A, residues 825–843 of human

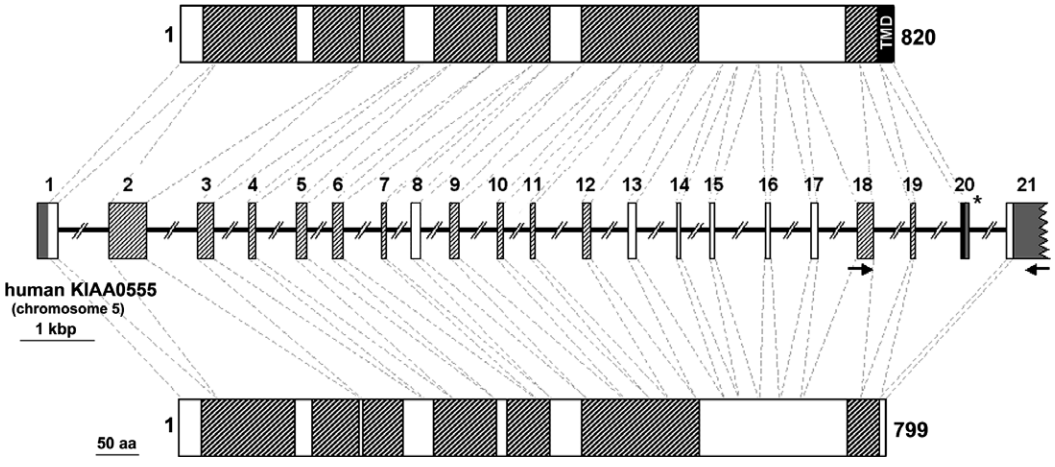

Fig. 2. Genomic organization and transcription of human KIAA0555 gene. Exons and introns of the human KIAA0555 gene are represented by boxes and solid bars. Exons are numbered (1–21) on top of each box. Exons encoding the coiled-coil regions, the transmembrane domain (TMD) and the untranslated region are represented by hatched, black and shaded boxes, respectively. Alternative spliced exon is marked with an asterisk. Arrows below the exons stand for primers used to amplify the two KIAA0555 transcripts. Structure and number of amino acids predicted for both long and short KIAA0555 isoforms are indicated.

KIAA4091). Furthermore, the predicted membrane topology of KIAA4091 was also identical to that of KIAA0555. As that found for KIAA0555, no signal peptide sequence was detected in the KIAA4091 sequence.

The second KIAA0555 related gene codes for a 626 amino acid protein (GenBank Accession number NP_653321), which has been the focus of two recent studies and referred to as Marlin-1/Jamip1 [14,15]. Human KIAA0555 and Marlin-1/Jamip1 proteins show an identity of 58%. The C-terminal region of Marlin-1/Jamip1 is shorter than that in KIAA0555 (21 vs. 228 residues) and lacks the putative TMD.

3.5. Tissue distribution of KIAA0555 and KIAA4091 mRNAs

RT-PCR analysis confirmed that KIAA0555 gene is expressed in frog pituitary (Fig. 3A), from which its sequence was originally isolated, as well as in the rat and human gland (Fig. 3B and C). KIAA0555 was also expressed in other endocrine organs of these three species including the adrenal gland and testis. Besides the endocrine system, KIAA0555 mRNA was essentially detected in the central nervous system (CNS), which indeed exhibited expression levels higher than endocrine organs. A detailed analysis of KIAA0555 distribution in the rat and frog CNS revealed that it is highly expressed throughout the brain (Fig. 3A and B). In contrast, KIAA0555 was weakly expressed in peripheral tissues and was only detected in heart, liver and spleen.

To test whether alternative splicing of KIAA0555 gene could occur, we designed RT-PCR primers flanking the nucleotide sequence coding for the TMD of KIAA0555 (Fig. 2). As predicted, two PCR products with the expected size were detected in frog, rat and human (Fig. 3A–C), whose sequencing confirmed that alternative splicing generates two distinct mRNAs differing in the presence or absence of the sequence coding for the TMD. Furthermore, PCR analysis showed that KIAA0555 gene is alternatively spliced in a tissue-specific manner so that CNS expressed exclusively the long isoform, whereas both isoforms were present in endocrine organs (Fig. 3A–C). In most peripheral tissues, the long isoform of KIAA0555 was preferentially expressed over the short isoform.

RT-PCR analysis of rat and human tissues revealed that, similar to that found for KIAA0555, KIAA4091 gene was predominantly expressed in the CNS and endocrine tissues, and

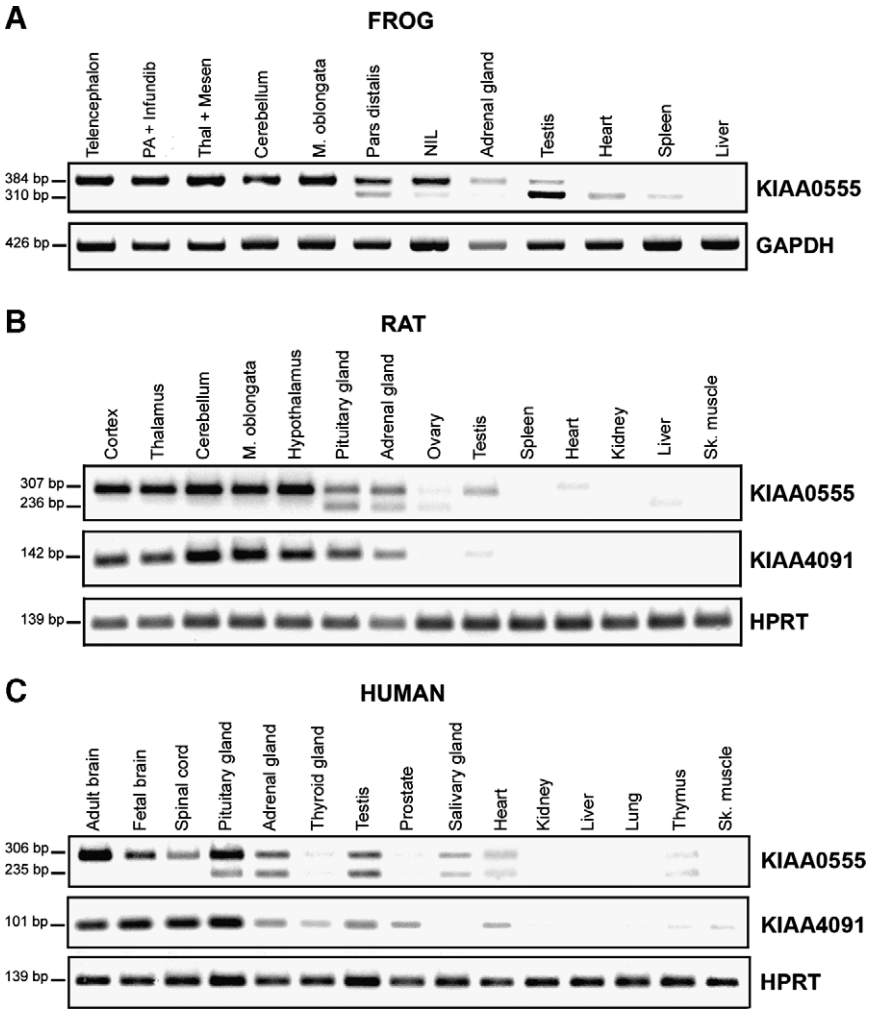

Fig. 3. Expression of KIAA0555 and KIAA4091 transcripts in frog (A), rat (B) and human (C) tissues. RT-PCR analysis of KIAA0555 mRNA species was performed using primers flanking the exon coding for the TMD. Total mRNA levels for KIAA4091 were determined by RT-PCR. HPRT and GAPDH amplification were used as internal controls for analysis of human and rat tissues and of frog tissues, respectively. The size of each product is shown. Sk. muscle, skeletal muscle; M. oblongata, medulla oblongata; PA, preoptic area; Infundib, infundibulum; Thal, thalamus; Mesen, mesencephalon; NIL, neurointermediate lobe.

showed low expression levels in other tissues such as heart, kidney, thymus or skeletal muscle (Fig. 3B and C). Amphibian KIAA4091 was not included in this analysis, as its cDNA sequence is still unknown.

Taking into account the structural characteristics and expression profiles of KIAA0555 and KIAA4091 genes, we will hereinafter refer to them as NECC1 and NECC2 (neuroendocrine long coiled-coil proteins 1 and 2), respectively.

3.6. Subcellular distribution of NECC proteins

The subcellular localization of NECC proteins was investigated by transfecting the corresponding GFP-fusion proteins in HEK293 AD cells. Examination of the transfected cells by confocal fluorescence microscopy revealed that both NECC1 isoforms as well as NECC2 accumulate in yuxtanuclear structures displaying tubulo-vesicular shape (Fig. 4A). Immunostaining of transfected cells with an antibody against GM130 showed no co-localization of GFP-tagged proteins with this *cis*-Golgi marker, yet GFP-labeled structures were found in close proximity to those immunostained with the anti-GM130 antibody (Fig. 4A). To further investigate the identity of the intracellular compartments wherein exogenously expressed NECCs accumulate, we used the ts045 variant of the vesicular stomatitis virus glycoprotein conjugated to GFP (VSVG-GFP ts045). VSVG ts045 has been widely used to study membrane transport because of its reversible misfolding and retention in the endoplasmic reticulum (ER) at 40 °C while when the incubation temperature is shifted to 32 °C, VSVG ts045 synchronously exits the ER and traverses the Golgi complex in route to the plasma membrane. Specifically, accumulation of VSVG ts045 at the Golgi complex occurs after 30 min at 32 °C [16]. Examination of double transfected cells by confocal microscopy showed no co-localization of VSVG-GFP ts045 and cMyc-tagged NECC proteins at the ER (data not shown). In contrast, partial co-localization of the exogenously expressed NECC proteins with VSVG-GFP ts045 was observed at the Golgi complex (Fig. 4B).

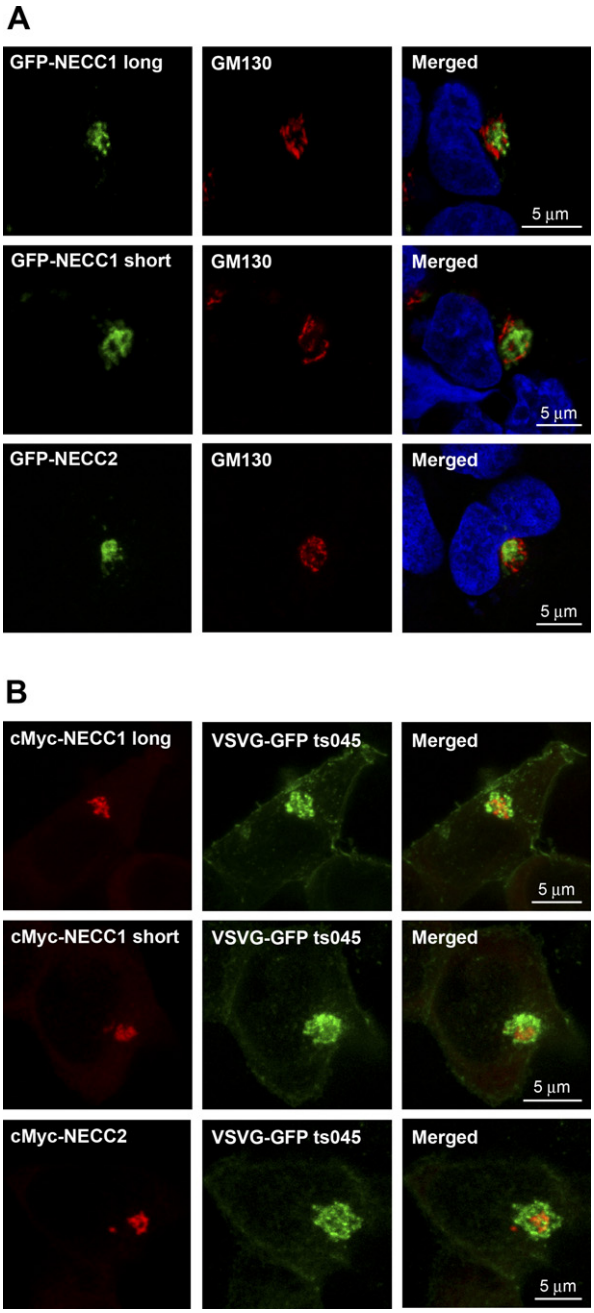

Fig. 4. Co-localization of NECC recombinant proteins with markers of the Golgi complex in transiently transfected HEK293 AD cells. (A) Confocal microscopy images of cells transfected with GFP-NECC1 long isoform, GFP-NECC1 short isoform or GFP-NECC2 (green) and immunostained against GM130 (red). Nuclei are shown by DAPI staining (blue). (B) Confocal microscopy images of cells co-transfected with cMyc-NECC1 long isoform, cMyc-NECC1 short isoform or cMyc-NECC2 (red) and the VSVG-GFP ts045 vector (green). Cells were incubated overnight at 40 °C, then shifted at 32 °C for 30 min and immunostained against the cMyc epitope tag.

4. Discussion

To identify genes involved in the control of the regulated secretory pathway, we applied a Differential Display methodology using two frog melanotrope subtypes displaying opposite secretory phenotypes. This analysis has led to the identification of an uncharacterized sequence preferentially expressed in cells displaying low secretory activity which, based on its structural features and tissue expression pattern, was referred to as NECC1 (neuroendocrine long coiled-coil protein 1). This gene belongs to a family consisting of two additional members, Marlin-1/Jamip1 [14,15], and a new gene of unknown function referred by us to as NECC2 on the basis of its structural and expression similarities to NECC1. The gene family including NECC1, NECC2 and Marlin-1/Jamip1 was only found in vertebrate genomes. Both NECC1 and NECC2 amino acid sequences show a high conservation degree along vertebrate evolution, thereby pointing out the likely physiological relevance of these genes in vertebrates.

NECC1 and 2 proteins display a similar structural organization. Thus, both proteins are predicted to contain several coiled-coil regions (7 for NECC1 and 8 for NECC2) and a TMD at their C-terminus. Marlin-1/Jamip1 exhibits 4 coiled-coil regions but lacks the TMD [15]. This protein family does not show common amino acid signatures for characterized protein families. However, the existence of multiple coiled-coil regions spanning almost the whole length of their amino acid sequences is also present and defines a heterogeneous group of protein families named as long coiled-coil proteins. Long

coiled-coil domains of up to several hundred amino acids usually form 'rod'-like tertiary structures and are found in a variety of proteins displaying distinct functions such as the components of intermediate filaments of the cytoskeleton and nuclear lamina, or the molecular motors interacting with the actin cytoskeleton (i.e. myosin) or with microtubules (i.e. kinesin and dynein) [17,18].

In addition, a group of long coiled-coil proteins has been reported to participate at different steps of the secretory pathway [19] including golgins, associated to the Golgi Complex [20], the endosome-related proteins EEA1, Rabaptin-5 and Rabip4 [21], or the components of the cytomatrix at the presynaptic active zone CAST and liprin-α proteins [22]. In view of their ability to form long rod-like molecules, these long coiled-coil proteins have been proposed to act as tethering factors, either for the maintenance of organelle structure or for bringing transport vesicles close to the membrane of the target organelle prior to membrane fusion [19]. Furthermore, as that found for NECC proteins, most long coiled-coil proteins participating in the regulation of membrane traffic contain some small stretches of non-coiled-coil sequences interspersed among the coiled-coil which would act as hinges enabling vesicle docking [19].

Most long coiled-coil proteins participating in the secretory pathway present specific regions at their N- or C-termini mediating organelle-specific targeting. Interestingly, in addition to the existence of multiple coiled-coil regions, NECC proteins show a putative TMD at their C-terminus which, due to its relatively short length (19 amino acid residues), would likely anchor these proteins to cellular bilayers of reduced thickness such as the Golgi membranes [23]. This has been proven to be the case for some members of the golgin family of proteins located at the *cis*-side of the Golgi Complex (i.e. giantin, golgin-84 and CASP), which also bear a 19-amino acids transmembrane domain at their C-terminus [24]. Notwithstanding this, NECC proteins and golgins containing TMD exhibit differences in the sequence of their TMDs; specifically, the TMD of NECC proteins lacks the conserved tyrosine and histidine residues which are present in the TMD of CASP, giantin and golgin-84 [24].

When viewed together, the structural characteristics of NECC1 and NECC2 sequences suggest that these proteins could be included in the group of long coiled-coil proteins participating in the secretory pathway. Furthermore, co-transfection studies using the membrane traffic marker VSVG-GFP ts045 and NECC expression vectors revealed that exogenously expressed NECC proteins localize at the Golgi complex and, specifically, at distal compartments of this organelle, since no co-localization was found between these proteins and the *cis*-Golgi matrix protein GM130. Based on these results, we propose these proteins as new members of the golgin family. Accordingly, NECC proteins could play a role comparable to that reported for known golgins such as the maintenance of Golgi structure and/or the regulation of membrane fusion events. Moreover, in view of the preferential expression of NECC1 in melanotropes featuring a hyposecretory phenotype, it is tempting to propose an inhibitory role for NECC1 in intracellular transport. In line with this notion, Barr et al. have suggested that golgins can inhibit membrane trafficking by impairing membrane fusion events keeping membranes apart and/or providing transient binding sites for vesicles to limit their diffusion [19]. In addition, the preferential expression of NECC1 and NECC2 in tissues containing cells with regulated secretory pathway (i.e. CNS and endocrine glands) further supports a role for NECC proteins in the regulated secretion. Notwithstanding this, we also detected low levels of NECC1 and 2 transcripts in heart or salivary gland. This can be related to the existence of cells with regulated secretion in these organs, such as the atrial cardiocytes producing atrial natriuretic peptide [25], or parotid cells, which release salivary proteins by regulated secretion [26].

We cannot exclude the possibility that NECC1 and 2 play roles similar to those suggested for the third member of this family, Marlin-1/Jamip1, which include the control of the intracellular transport of membrane receptors [14]. Undergoing studies in our laboratory are aimed to determining the specific location and functions of NECC proteins in (neuro)endocrine cells.

The finding that the short isoform of NECC1 is exclusively expressed in endocrine cells but not in the brain indicates that this gene is alternatively spliced in a tissue-specific manner and suggests that the two isoforms could play, at least partially, distinct functions. In fact, the short isoform likely codes for a soluble, cytoplasmic protein. As mentioned before, our confocal microscopy studies support the view that this protein, as the other NECCs, is linked to Golgi compartments thus suggesting that it might interact with Golgi-associated proteins, as has been proposed for other golgins lacking a TMD [19]. Taking into account the ability of long coiled-coil proteins to dimerize and the similar intracellular distribution of the two isoforms of NECC1, it is also plausible that these two proteins interact in endocrine cells by forming dimers, which, in turn, would recruit the NECC isoform lacking the TMD to the compartment(s) wherein the long isoform of NECC1 is anchored by virtue of its TMD. As has been proposed for the long coiled-coil protein involved in membrane traffic GMAP210, for which alternative spliced isoforms have been also reported, dimerization of NECC1 isoforms could serve as a mechanism to fine tune the function of these proteins within the cell [27]. Future studies will be aimed at investigating whether interaction of endogenous NECC1 proteins do occur in endocrine cells.

In conclusion, we have identified two new proteins, structurally related to the golgin family, which may play important roles in the control of the regulated secretory pathway.

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