Identification and characterization of homologues of the Exocyst component Sec10p

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Abstract **The *SEC10* gene product is a member of the Exocyst complex essential for exocytosis in the budding yeast *Saccharomyces cerevisiae*. We report here the cloning and characterization of human Sec10p (hSec10p; GenBank accession number U85946). hSec10p is a 77-kDa protein with 23% amino acid identity to yeast Sec10p and 37% identity to a *C. elegans* protein found in the database. Northern and Western blot analyses indicate that hSec10 has a broad tissue distribution. Immunofluorescence staining of COS cells cotransfected with hSec10p and a mammalian Sec8p demonstrates that these two proteins have an identical distribution in the cell including a localization in the peripheral cytoplasm. These data suggest that hSec10p is a component of the mammalian counterpart of the yeast Exocyst complex essential for post-Golgi traffic.**

Key words: Exocytosis; Exocyst; Sec10p

1. Introduction

Exocytosis is essential for eukaryotic cells by providing the basis for fundamental processes such as cell growth or cell-cell communication. The secretory pathway is organized according to the same principles in all eukaryotic cells. Membrane or luminal cargoes are transported from one intracellular donor compartment to the next acceptor compartment via vesicular traffic until the final destination is reached. A complex protein machinery has evolved that ensures the orderly flow of transport vesicles to their appropriate destinations in the cell (for review, see [1]). This transport machinery is highly conserved in all eukaryotes [2]. Elucidation of the proteins involved at each transport step and their interplay is the focus of intense research. A number of different approaches in a variety of cell types have been undertaken to identify these proteins.

Genetic analysis of exocytosis in the budding yeast *Saccharomyces cerevisiae* has revealed a number of gene products that are important in exocytosis [3]. Recently, it was shown that Sec3p, Sec5p, Sec6p, Sec8p, Sec10p and Sec15p as well as a novel gene product named Exo70p form a 19.5S complex in the cells [4–6]. This complex was named the Exocyst [7]. Immunofluorescence staining shows that the complex is specifically concentrated at the tip of small buds, the active site of exocytosis in yeast [5]. Recently, mammalian homologues for Sec6p and Sec8p have been identified. Like their yeast counterparts, they are components of a high molecular weight complex in rat brain [8,9].

In this study we describe the cloning of a human homologue of the essential yeast secretory protein Sec10p. This protein has 23% amino acid identity to yeast Sec10p and 37% identity to a *C. elegans* protein found in the database. Further characterization of the human Sec10p suggests that it is a component of the mammalian Exocyst complex.

2. Materials and methods

2.1. cDNA cloning and sequence analysis of hSec10

A search of the dbEST database using yeast Sec10p sequence has identified a peptide encoded by a human expressed sequence tag (EST; GenBank accession number H96462) that is homologous to yeast Sec10p and a putative *C. elegans* protein encoded by gene C33H5.9 (GenBank accession number U41007) [6]. Polymerase chain reaction (PCR) primers were designed based on the human EST sequence and a PCR product was generated from a human cerebellum cDNA library (Clonetech). The PCR product was ^{32}P-labeled using a random primed DNA labeling kit (Boehringer Mannheim) and used to screen for the full-length cDNA of the human homologue of Sec10 (hSec10). Sequence comparisons were performed using the BESTFIT program (Genetics Computer Group). The gap weight was 3.00 and the length weight was 0.10. The sequences were randomized and the Z values (Z=(quality score of alignment−mean quality score of 15 alignments)/standard deviation) were calculated.

2.2. Northern blot analysis

A human multiple tissue blot containing approximately 2 μg of poly(A$^+$) RNA per lane from eight different human tissues (Clonetech) was hybridized with the ^{32}P-labeled 1.6-kb *Eco*RI fragment of hSec10 (nucleotides −198–1404) or a rSec6 fragment (nucleotides 1–1679) using ExpressHyb hybridization solution (Clonetech) according to the manufacturer's instructions.

2.3. Western blot analysis

A DNA fragment containing nucleotides 1–1404 of hSec10 cDNA was generated by PCR and subcloned by *Eco*RI sites into pGEX4T-1 vector (Pharmacia) and pTrcHisB vector (Qiagen) and expressed as a glutathione *S*-transferase (GST) fusion protein and histidine-tagged recombinant protein, respectively. The GST fusion protein was purified using glutathione Sepharose and injected into rabbits for antibody production. The antibody was affinity-purified using histidine-tagged recombinant protein transferred to nitrocellulose. For Western blot analysis, rat tissues were homogenized in 25 mM Tris-HCl, pH 7.4, 0.3 M sucrose, 120 mM NaCl, 1 mM EDTA containing 0.2 mM phenylmethylsulfonyl fluoride, 0.5 μM pepstatin A, 0.1 μM aprotinin, and 1.0 μM leupeptin. The homogenates were centrifuged at 1000×g and the supernatants (200 μg/lane) were analyzed by Western blotting using affinity-purified hSec10 antibody.

2.4. Expression and immunofluorescence localization of hSec10 and mSec8 in transfected COS-7 cells

A PCR primer was designed that encodes the C-terminus of hSec10p and the peptide sequence of c-*myc* that can be recognized by the 9E10 monoclonal antibody. This primer was used in conjunction with N-terminal hSec10 primers to produce the modified hSec10 cDNA fragment and subcloned by *Eco*RI and *Xho*I sites into the pcDNA3 expression vector (Invitrogen). A mouse Sec8 (mSec8)

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cDNA was kindly provided by Dr. Philippe Soriano (Baylor College of Medicine, Houston, TX). To construct HA-tagged mSec8, a primer containing the oligo encoding the HA epitope and the N-terminal sequence of mSec8 was used for PCR. The DNA product was subcloned into pcDNA3 by *Hin*dIII sites. For eukaryotic expression, COS-7 cells were cultured on poly-L-ornithine-coated coverslips in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and 100 U/ml each of penicillin and streptomycin. The expression constructs were transfected into the cells using LipofectAMINE reagent (Gibco BRL). The transfected cells were fixed with 4% formaldehyde in 0.1 M phosphate buffer (pH 7.5), and immunostained for microscopic observation. The HA-tagged mSec8p was detected with a rabbit polyclonal antibody against the HA epitope sequence (BAbCO). The c-*myc*-tagged hSec10p was detected with 9E10 monoclonal antibody. Secondary staining was performed using FITC-conjugated goat anti-mouse IgG and CY3-conjugated goat anti-rabbit IgG (Jackson Immunochemicals), respectively.

3. Results

3.1. Cloning and sequence analysis of Sec10 homologues

A database search for homologues of the yeast post-Golgi trafficking proteins has been performed. A human brain EST (H96462) was found to encode a peptide that is homologous to the C-terminus of ySec10p [6]. By PCR and library screening, we have isolated cDNA clones encoding the full-length human homologue of ySec10p (hSec10p). The open reading frame of the clones contains 2127 nucleotides encoding a 708-amino acid protein with a predicted molecular weight of 80 kDa. Sequence comparison of hSec10p with ySec10p using BESTFIT revealed 23% identity and 50% similarity of the two proteins at the amino acid level (Fig. 1A). The quality score of the sequence was compared with the randomized sequences of hSec10p. This score was 23 standard deviations above the average score of the randomized sequences ($Z = 23$). Therefore, the homology between the human and the yeast sequences is highly significant and is not due to an overall similarity of their amino acid compositions. These sequence data are comparable or higher than those observed between the rat brain proteins rSec6p and rSec8p [8] and their corresponding yeast counterparts (Table 1). The full-length hSec10p sequence has also been used to search the yeast *Saccharomyces cerevisiae* database. The yeast protein in the database that was found to be most homologous to hSec10p was ySec10p.

In addition to identifying hSec10p, a potential *C. elegans* homologue of the yeast Sec10p has also been found in the database. The *C. elegans* gene C33H5.9 encodes a protein (cSec10p) of 659 amino acids and has a BLAST probability value of 8.5×10^{-14}. BESTFIT analysis shows that this protein shares 21% identity and 47% similarity with ySec10p. The sequence homology between hSec10p and cSec10p was also compared. These two proteins share 37% identity and 60.5% similarity. A sequence comparison of the three proteins is shown in Fig. 1B.

Sequence analyses of these homologues have also been performed. None of the homologues is predicted to be an integral membrane protein although several short stretches of hydrophobic residues are present in the C-terminal region. One interesting feature of hSec10p and cSec10p is that their N-terminal regions are predicted to form coiled-coil structures when analyzed using the Macstripe program [10]. The corresponding regions in yeast Sec10p were also predicted to form a coiled-coil structure [6]. The function of this region is unknown. It has been thought that the coiled-coil regions in the

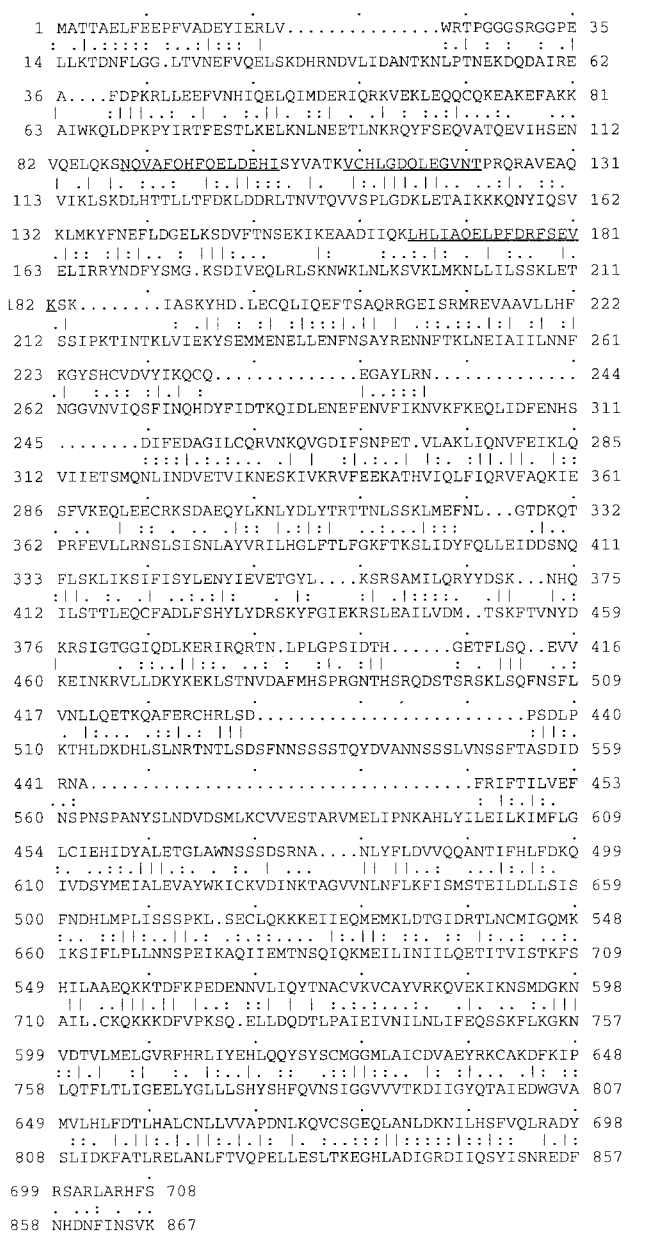

Fig. 1. A (see above): Alignment of protein sequences of yeast Sec10p (ySec10) and human Sec10p (hSec10). Sequences are aligned using the BESTFIT program (Genetics Computer Program). Vertical bars indicate identical residues and dashed lines indicate conserved amino acids. The underlined sequences indicate the matches of hSec10p to p71 peptide sequences (see text). B (see following page): Comparison of protein sequences of yeast *S. cerevisiae* Sec10p (ySec10), human Sec10p (hSec10), and *C. elegans* Sec10p (cSec10). Identical residues are demonstrated by black boxes, and conserved residues are indicated using half-filled boxes.

Exocyst members may be involved in protein-protein interactions [6].

3.2. Tissue distribution of hSec10

To examine the tissue distribution of the hSec10 mRNA, Northern blot analysis was performed using a blot that contains poly(A^+) RNA from different human tissues (Clonetech). As shown in Fig. 2, a major band of 4.5 kb was detected in all the tissues examined. In addition, a 7.3-kb band

Sequence Comparison of Sec10p Homologues

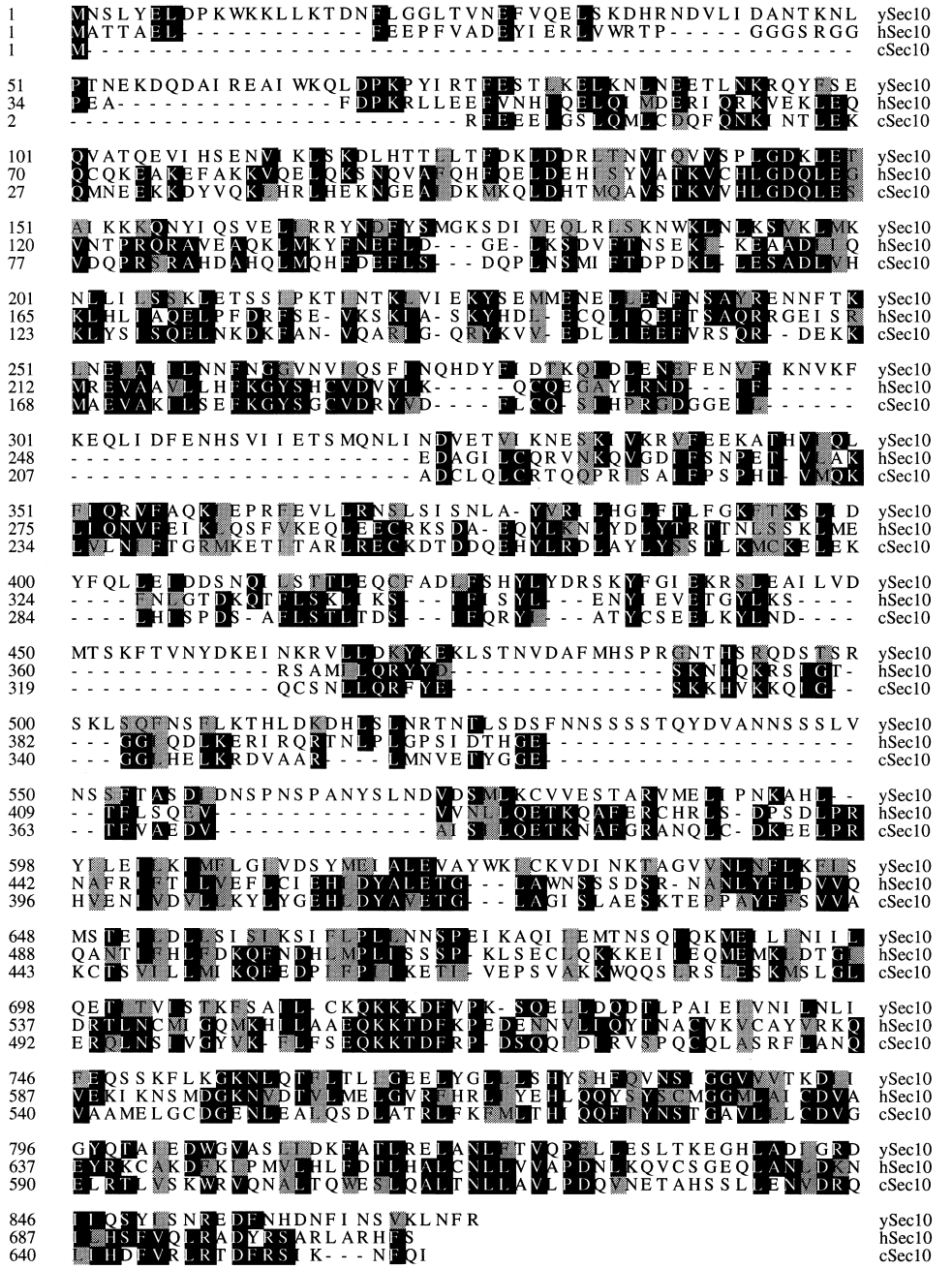

Fig. 1 (*continued*).

was also weakly detected, suggesting that there is another isoform of hSec10 in the tissues. After the detection of hSec10, the same blot was stripped and used to detect the rabbit Sec6 message [8]. A very similar pattern of tissue distribution was found (not shown).

The tissue distribution of hSec10p was also examined using

Table 1
Homology analyses of the known yeast Exocyst homologues

Homologue	Identity (%)	Quality score	Randomized average/SD	*Z* value
rSec6	22	301.9	238.0/5.6	11.4
rSec8	20	400.6	313.6/6.5	13.4
cSec10	21	289.3	221.2/5.2	13.1
hSec10	23	337.4	233.8/4.5	23.0

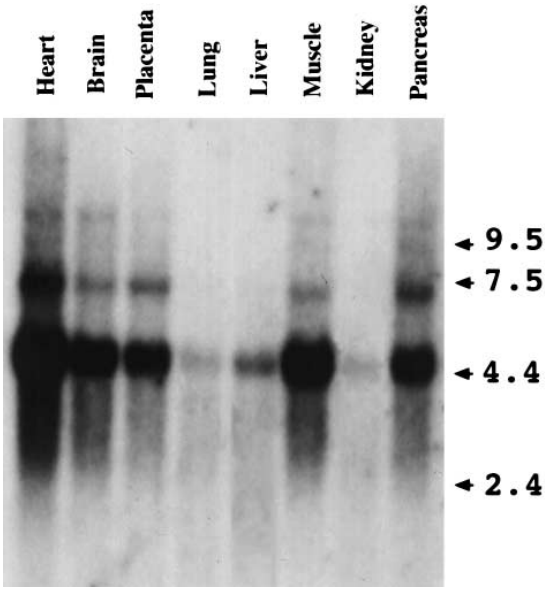

Fig. 2. Multiple-tissue Northern blot analysis of human Sec10 mRNAs. Each lane contains 2 μg of poly(A^+) RNA from different human tissues (Clonetech). Molecular size markers (kilobases) are indicated on the right.

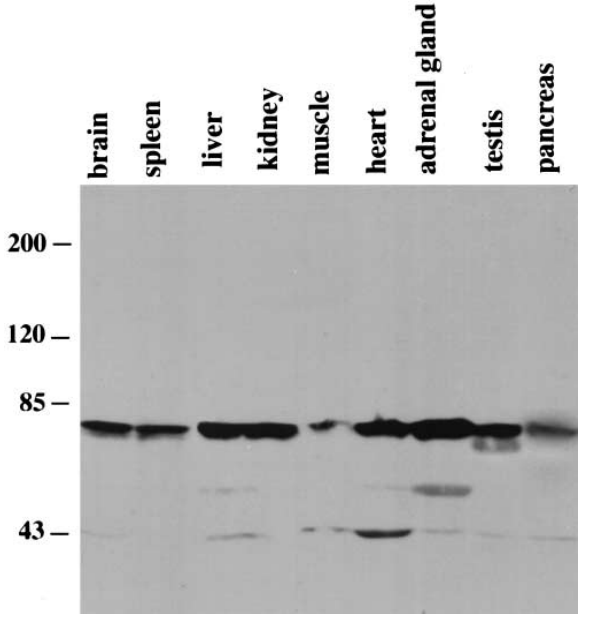

Fig. 3. Western blot analysis of hSec10 in various tissues. Each lane contains 200 μg of postnuclear supernatant from various rat tissues. The affinity-purified anti-hSec10 antibody was used for immunostaining.

an affinity-purified rabbit polyclonal antibody generated against the N-terminal portion of hSec10p. This antibody recognized a band of ~77 kDa, consistent with the predicted molecular weight of hSec10p. Western blot analysis of several rat tissues using this antibody indicated that Sec10p is present in all the tissues examined (Fig. 3). Higher amounts of this protein were found in liver, kidney, heart and adrenal glands.

3.3. Colocalization of mSec8 and hSec10 in the cells

In yeast, Sec10p is a member of the Exocyst complex [6]. Biochemical and genetic data suggest that Sec10p carries out its function in concert with other members of the Exocyst complex [5,6]. We examined therefore whether hSec10p colocalizes with the known members of the mammalian Exocyst complex in the cells. Since the available antibody did not recognize endogenous Sec10p by immunofluorescence, we compared the localization of epitope-tagged hSec10p and a mammalian homologue of Sec8p (mSec8p) in transfected cells. hSec10p and mSec8p were tagged by c-*myc* and HA epitopes, respectively, and cotransfected into COS-7 cells. The immunolocalization of the transfected proteins was examined using anti-c-*myc* monoclonal antibody and anti-HA polyclonal antibody. As shown in Fig. 4, the localizations of the two proteins are almost identical in the transfected cells. They are both widely distributed throughout the cytoplasm, and a colocalization was also evident in peripheral cytoplasmic regions consistent with their involvement in exocytosis. In yeast cells, the Exocyst members were found in the cytoplasm and specifically concentrated in the bud tip, an active site for exocytosis [5] (Finger et al., in preparation). The colocalization of hSec10p and mSec8 suggests that they function at the same sites in the cells.

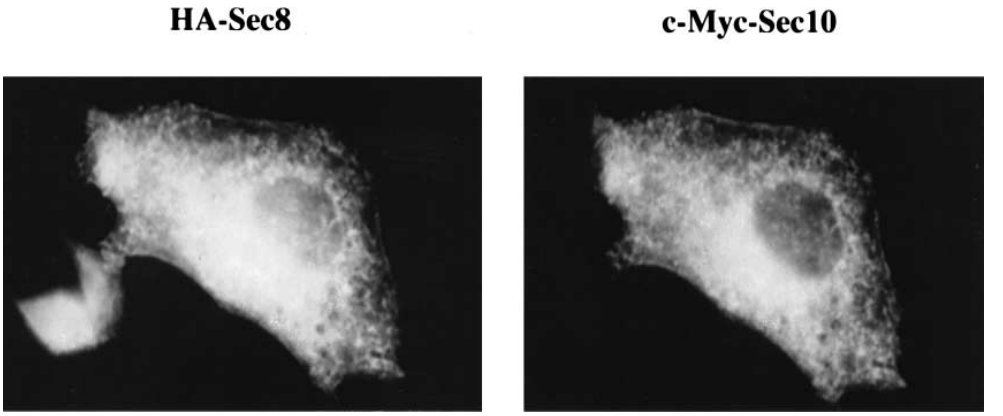

Fig. 4. hSec10 colocalizes with mSec8 in transfected COS-7 cells. COS-7 cells were cotransfected with HA-tagged mSec8 and c-*myc*-tagged hSec10. A polyclonal antibody against HA epitope and a monoclonal antibody against c-*myc* epitope were used for immunostaining.

4. Discussion

Exocytosis is a complex process involving a cascade of protein-protein interactions. The SNARE proteins (for review, see [1]) are thought to provide a recognition system which ensures that the secretory vesicles generated from Golgi are targeted to the plasma membrane rather than to other membranes in the cells. However, since the t-SNAREs are broadly distributed all over the plasma membrane whereas secretion is restricted to specific domains of the cell surface (for example, in neurons and epithelial cells), there must exist another layer of targeting specificity that cannot be solely conferred by SNARE interactions [11]. It is believed that additional proteins interact with the basic secretory machinery and probably work in conjunction with the cytoskeleton to direct the docking and fusion of secretory vesicles to the appropriate domains of the plasma membrane. What these regulatory proteins are, and how they define the temporal and spatial specificity of secretion, are clearly important questions in the field of protein trafficking. The Exocyst complex has been hypothesized to be involved in mediating this layer of specificity [6,12] because of its specific localization in the bud tip and its potential interactions with the SNAREs and the *rab* GTPase which participate in exocytosis [5,7,9]. Molecular cloning of members of the Exocyst from mammalian cells will provide us with the tools to understand the role of these proteins in a wide range of biological processes such as neurotransmitter release and epithelial cell differentiation.

Here we report the identification and characterization of homologues of an Exocyst component Sec10p in human brain and *C. elegans*. We find that these Sec10 homologues have similar overall protein structures to their yeast counterpart. In particular, all these proteins contain coiled-coil regions at their N-termini. In fact, all the known yeast and mammalian Exocyst components seem to contain coiled-coil structures [6,9]. These coiled-coil domains may contribute to the subunit assembly and stabilization of the complex. The human Sec10p has been further characterized in this study. Northern and Western blot analyses demonstrated that the mammalian Sec10p is broadly distributed in all the tissues examined, suggesting that it plays a fundamental role in all cells. In addition, hSec10p colocalizes with the mammalian Sec8p in transfected cells. Recently, a mammalian complex containing rSec6, rSec8 and several other proteins was purified from rat brain [9]. Peptide sequences of the proteins have also been reported. Interestingly, all three peptide sequences reported for p71 can be found within the hSec10p sequence (Fig. 1A, underlined sequences). This result indicates that hSec10p corresponds to the 71-kDa component of the purified mammalian Exocyst complex, confirming the prediction that our cloned hSec10p is part of a mammalian Exocyst complex. Our studies on hSec10p will provide the basis for future investigations aiming to understand the structural organization of the Exocyst components and its functional role in mammalian cells.

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