Introduction

Entry to the secretory pathway is initiated by the extrusion of COPII-coated cargo-loaded transport vesicles and tubules from the endoplasmic reticulum (ER). The basic outlines of COPII coat formation and the extrusion process are now understood in considerable detail (for reviews see Springer et al., 1999; Brittle and Water, 2000; Klumperman, 2000; Antonny and Schekman, 2001; Bickford et al., 2004). As first established in S. cerevisiae by genetic analysis, and later confirmed by biochemical and ultrastructural studies in both yeast and in other species, cargo sorting and vesicle budding from the ER is driven by three cytosolic complexes containing a total of five proteins: (1) the small GTP-binding protein Sar1p initiates coat formation at specialized ER exit sites (ERES) (Bannykh et al., 1996; Aridor et al., 2001); (2) the heterodimeric complex of Sec23-Sec24 binds to Sar1p-GTP and provides for cargo selection (Aridor et al., 1998; Miller et al., 2003), membrane bending (Bi et al., 2002), and the presumably binds Sec23/24. The Sec31B-T group (molecular mass 52,983 Da) contains a preserved WD-repeat domain but lacks the C-terminal proline-rich region. When expressed as a fusion protein with eYFP in cultured cells, Sec31B-F associates with the endoplasmic reticulum and with vesicular-tubular clusters, displays restricted intracellular movement characteristic of COPII vesicle dynamics, co-distributes on organelles with Sec13, Sec31A and Sec23 (markers of the COPII coat), and concentrates with ts045-VSV-G-CFP (VSV-G) when examined early in the secretory pathway or after temperature or nocodazole inhibition. The role of the truncated form Sec31B-T appears to be distinct from that of Sec31B-F and remains unknown. We conclude that Sec31B-F contributes to the diversity of the mammalian COPII coat, and speculate that the Sec31 cage, like Sec24, might be built with isoforms tuned to specific types of cargo or to other specialized functions.

Summary

We have cloned human brain and testis Sec31B protein (also known as secretory pathway component Sec31B-1 or SEC31-like 2; GenBank accession number AF274863). Sec31B is an orthologue of Saccharomyces cerevisiae Sec31p, a component of the COPII vesicle coat that mediates vesicular traffic from the endoplasmic reticulum. Sec31B is widely expressed and enriched in cerebellum and testis. Its predicted sequence of 1180 residues (expected molecular mass 128,711 Da) shares 47.3% and 18.8% similarity to human Sec31A (also known as Sec31; GenBank accession number AF139184) and yeast Sec31p, respectively. The gene encoding Sec31B is located on chromosome 10q24 and contains 29 exons. PCR analysis of exon utilization reveals massive alternative mRNA splicing of Sec31B, with just 16 exons being constitutively utilized in all transcripts. The presence of a stop codon in exon 13 generates two families of Sec31B gene products (each displaying additional patterns of mRNA splicing): a group of full-length proteins (hereafter referred to as Sec31B-F) and also a group of truncated proteins (hereafter referred to as Sec31B-T), distinguished by their utilization of exon 13. Sec31B-F closely resembles Sec31p and Sec31A, with canonical WD repeats in an N-terminal domain that binds Sec13 and a proline-rich C-terminal region that presumably binds Sec23/24. The Sec31B-T group (molecular mass 52,983 Da) contains a preserved WD-repeat domain but lacks the C-terminal proline-rich region. When expressed as a fusion protein with eYFP in cultured cells, Sec31B-F associates with the endoplasmic reticulum and with vesicular-tubular clusters, displays restricted intracellular movement characteristic of COPII vesicle dynamics, co-distributes on organelles with Sec13, Sec31A and Sec23 (markers of the COPII coat), and concentrates with ts045-VSV-G-CFP (VSV-G) when examined early in the secretory pathway or after temperature or nocodazole inhibition. The role of the truncated form Sec31B-T appears to be distinct from that of Sec31B-F and remains unknown. We conclude that Sec31B-F contributes to the diversity of the mammalian COPII coat, and speculate that the Sec31 cage, like Sec24, might be built with isoforms tuned to specific types of cargo or to other specialized functions.

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Key words: ER, Secretory pathway, COPII, Vesicle, Golgi, Secretion, Protein isoforms

Human Sec31B: a family of new mammalian orthologues of yeast Sec31p that associate with the COPII coat

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also exit the ER in a similar manner (Siddiqi et al., 2003; Shoulders et al., 2004). GPI-linked proteins exit by yet a third unique pathway that involves their sorting into sphingolipid-rich rafts in the ER (Muniz et al., 2001). The cystic fibrosis transmembrane conductance regulator (CFTR) exits the ER by a COPII-dependent pathway and transits to the Golgi network in a non-conventional way, possibly trough an endosomal compartment (Yoo et al., 2002). Spectrin and ankyrin, cytoskeletal proteins that are absent in yeast, play a role in mammalian cells in determining the efficiency of transport of selected proteins through the secretory pathway, including even the efficiency of their exit from the ER and their pathway of intracellular targeting and/or retention (Devarajan et al., 1997; Tuvia et al., 1999; De Matteis and Morrow, 2000; Pradhan and Morrow, 2002). Such findings suggest that evolution has modified and added to the basic ER-exit and -sorting formula found in yeast, to meet the more complex and specialized needs of higher organisms. One way evolution modifies function is to develop protein orthologues and isoforms, e.g. by gene duplication and/or by alternative mRNA splicing. Both mechanisms appear to have been used in refining the mechanisms of ER export.

Mammalian orthologues have been identified for each of the five proteins involved in COPII coat formation, and in some cases, multiple isoforms of these proteins exist. For example, two mammalian isoforms of Sar1 and Sec23, and four mammalian isoforms of Sec24 have been reported (Kuge et al., 1994; Paccaud et al., 1996; Pagano et al., 1999; Tang et al., 1999). Only one form of mammalian Sec13 has been described (Shaywitz et al., 1995). Two groups have independently identified the mammalian protein corresponding to the Sec31p yeast protein, named Sec31A (Shugrue et al., 1999; Tang et al., 2000). It has also been noticed, by examining published expressed sequence tags (ESTs), that a second mammalian orthologue of Sec31p, named Sec31B, exists that has not yet been characterized (Tang et al., 2000). With the exception of a putative role in mediating cargo specificity (Shimoni et al., 2000), and a single report of Sec23-Sec24 participation in endocytosis (Penalver et al., 1999), no specific role for isoform Sec31B-T. The full-length gene products are similar to human Sec31A and with the molecular mass of 128,711 Da (Fig. 1). A satisfactory Kozak initiation sequence is present immediately upstream of the first ATG and an in-frame stop codon occurs at nt 93. We are thus confident that the second ATG at nt 99 is the initiator methionine of the derived protein. We have termed the derived protein from this transcript Sec31B-F, in recognition of its full-length structure, and the existence of shorter truncated peptides that arise by alternative exon usage from this gene (see below).

In the present study we have cloned and characterized human Sec31B and determined the intron-exon structure of the gene encoding Sec31B (also known as Sec31L2). We present evidence that, in addition to being widely expressed in mammalian tissues, mRNA transcripts of Sec31B (also known as secretory pathway component Sec31B-1 or Sec31-like 2; GenBank accession number AF274863) display considerable alternative mRNA splicing, generating a range of highly divergent protein products that fall into two general categories, several alternative versions of full-length Sec31B1 proteins with a mass of about 129 kDa, and a group of truncated proteins including one of about 53 kDa (hereafter referred to as Sec31B-T). The full-length gene products are similar to Sec31A1 in structure, disposition and their apparent function, and appear to be bona fide components of the mammalian COPII coat. The 53 kDa gene product assumes an intracellular distribution distinct from Sec31A1. Its role remains unknown and will be addressed in future studies. The focus of the present study is on a full-length Sec31B protein (hereafter referred to as Sec31B-F).

Results

Identification of human Sec31B and the exon structure of Sec31B

The gene for Sec31B was first identified in silico by pair-wise comparison of rat Sec31 aa sequences (Shugrue et al., 1999) with the high-throughput genomic-sequence-database using TBLASTN (http://www.ncbi.nlm.nih.gov/BLAST). A match was made with GenBank Homo sapiens chromosome 10, clone RP11-411B6 (GenBank accession number AL133352) deposited by the Sanger Centre. In addition, 38 sequence matches were identified from the dbESTs, of which five were from both testis and ovary libraries. One EST matched a partial clone (accession number AL080141) in the non-redundant nucleotide database submitted by the German Cancer Research Center. Exon mapping of adjacent genomic sequences allowed identification of WNT8B as an adjacent gene; this allowed mapping of Sec31B to locus 10q24. Oligonucleotides were designed to amplify contigs using Marathon-Ready cDNA libraries from human testis, brain and fetal brain. Sequence at the 5'-end was extended by RACE amplification. The oligonucleotides used to span the sequence of Sec31B are summarized in supplementary material Fig. S1. All reaction products from each library were bi-directionally sequenced (Keck Biotechnology Laboratories, Yale University) from at least three independent amplifications to eliminate PCR-derived errors. All libraries demonstrated an abundance of alternative transcripts (see below). The full-length cDNA transcript identified from this analysis and identified as Sec31B, was deposited under the GenBank accession number AF274863. This full-length cDNA comprises 4648 nucleotides (nts), and includes 5'- and 3'-untranslated regions of 98 and 1010 nts, respectively. The open-coding-region consists of 3540 nts, predicting a 1180 amino acid (aa)-long protein with the molecular mass of 128,711 Da (Fig. 1). A satisfactory Kozak initiation sequence is present immediately upstream of the first ATG and an in-frame stop codon occurs at nt 93. We are thus confident that the second ATG at nt 99 is the initiator methionine of the derived protein. We have termed the derived protein from this transcript Sec31B-F, in recognition of its full-length status and the existence of shorter truncated peptides that arise by alternative exon usage from this gene (see below).

A comparison of the predicted aa sequence of Sec31B-F with human Sec31A and with S. cerevisiae Sec31p reveals the greatest degree of homology over the N-terminal half of the protein, the region that contains seven canonical WD repeats. Its homology to Sec31p is reduced in the proline-rich regions distributed over its C-terminal half (sequences involved with the binding of Sec23). Overall, Sec31B-F shares 47.3% and 18.8% identity to human Sec31A and yeast Sec31p, respectively.

Search of the genome database with the complete Sec31B sequence revealed a match with the Sanger Centre Homo sapiens clone RP11-411B6 (GenBank accession number AL133352) and allowed its genomic structure to be deduced (Fig. 2). The gene encoding Sec31B is located at chromosomal locus 10p24, incorporates 29 exons, and spans 33,187 bp. As described below, the gene is subject to considerable alternative exon utilization. Of the 29 exons, 16 were found in all transcripts; these exons appear to be constitutively expressed and are depicted as green in Fig. 2. The other 13 exons (represented in red) are quite variably expressed. Exon 19 has
two putative splice-donor sites, verified by their presence in identified transcripts, and is therefore represented as 19A (closed) and 19B (open). Each intron-exon junction of human Sec31B is displayed in Fig. 2 with 20 base pairs of flanking sequence. The pattern of exon utilization of the Sec31B isoform as represented by GenBank (AF274863) is presented in Fig. 2C. This is the longest transcript observed experimentally. Also depicted are the exons translated to generate the two antibodies (Mab 1D4 and Mab 1G10) used in this study, both directed against sequences derived from exons 5 to 13, IgG 2013 directed against sequences from exons 16 and 17, and IgY 1871, directed to sequences derived from exons

Fig. 1. Sequence alignment of human Sec31B-F. The derived aa sequence of the human Sec31B-F (GenBank accession number AF274863) is compared with human Sec31A (Tang et al., 2000) and Sec31p (Salama et al., 1997) by the program MegAlign™ (DNAStar) using the J. Hein algorithm. Identical residues are shaded. Sec31B shares 47.3% and 18.8% identity to human Sec31A and yeast Sec31p respectively. The bar identified transcripts, and is therefore represented as 19A

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**Journal of Cell Science**

Shaywitz et al., 1997.

the N-terminal half of the protein, responsible for Sec13 binding, but lacks the C-terminal region responsible for Sec23-Sec24 binding.
Human Sec31B generates multiple transcripts by alternative mRNA splicing

Three cDNA libraries were exhaustively examined for alternate exon utilization by PCR (supplementary material Fig. S1). The Sec31B-F transcript (AL133352) utilized 26 of the 29 exons in the gene (Fig. 2C). Other exons not included in AL133352 were submitted separately to GenBank: exon 12, AF279137; exon 13, AF279138; exon 19B, AF279139 (only detected once, representing an alternative splice-donor at the 3’-end of exon 19); exon 21, AF279140. The frequency of observation of each exon in the transcripts examined is presented schematically in Fig. 3A. These frequencies also parallel closely the frequency with which such transcripts are observed in EST data available in GenBank. An area of micro-diversity was noted at the 3’ splice junction of exon 5, in which one codon would be deleted. This was detected in seven of 16 independent amplimers. Examples of four alternatively spliced

Fig. 2. Genomic structure of human Sec31B. The gene is located on chromosome 10p24, spans 33,187 bp and incorporates 29 exons. (A) Constitutively expressed exons are depicted as green boxes; alternatively utilized exons as red boxes. The size of each exon is drawn to scale. Arrows indicate the start-codon in exon 2 and the stop-codon in exon 29; these sites are utilized in Sec31B-F. Exon 19 has two putative splice-donor sites and is therefore represented as 19A (closed) and 19B (open). (B) Each intron-exon junction of human Sec31B is displayed with 20 base pairs of flanking sequence. The first exon begins with the adapter primer used for 5’ RACE and the last exon ends with a polyA tail. Lower- and upper-case letters denote intron and exon sequences, respectively (colored as above). (C) Exon utilization of the Sec31B-F isoform as represented by GenBank (AF274863). This is the longest transcript observed experimentally, predicted to encode a 129 kDa protein. Also depicted are the exons that had been translated to generate the antibodies used in this study. Mab1D4 (shown) and Mab1G10 were both raised against the same exons.
transcripts depicting their exon utilization are shown (Fig. 3B), together with a representation of their open reading frames.

Of significant interest was the frequent utilization of exon 13. This exon, detected in 23 of 27 transcripts that flanked this region, contains an in-frame stop codon; its incorporation is predicted to generate a truncated protein product with the molecular mass of 52,983 Da. The predicted translation products of Sec31B thus fall into two general categories. Those without exon 13 are expected to generate a family of alternatively spliced ‘full-length’ proteins (e.g. Sec31B-F). Conversely, transcripts incorporating exon 13 will generate truncated protein products not larger than 53 kDa. We term this latter group of ‘truncated’ proteins Sec31B-T, as represented in three of the four transcripts depicted in Fig. 3B. Because the reading frame downstream of the stop codon in exon 13 is open, we cannot formally exclude the possibility that proteins also exist that are generated by utilization of initiation sites other than the one in exon 2. Although there is some indication by western blotting that alternative translation initiation sites might be utilized (see Fig. 4), we have no conclusive evidence that this is indeed the case. It is also noteworthy that, when exon 4 is not utilized, then exon 5 contributes an out-of-frame stop codon. Thus, much shorter Sec31B peptides might also exist (because exon 4 is not constitutively utilized but exon 5 is). However, we have no other direct evidence for such very short expression products.

Although it is not possible in the absence of full-length mRNAs to determine the precise combinatorial patterns of exon utilization that actually occur, it is clear that many variations exist. Fig. 3C presents a cartoon summarizing the patterns of exon utilization that have been experimentally observed to date, along with the approximate location of the WD-repeat domains (present in both Sec31B-F and Sec31B-T) responsible for binding Sec 13, and the proline-rich domain (>24%) responsible for binding Sec23 (Shaywitz et al., 1997). This latter region is deleted in all proteins derived from Sec31B-T transcripts.

Both Sec 31B-F and Sec31B-T are widely expressed in tissues and cell lines by northern analysis, western blot, and by indirect

![Diagram A](image)

**A**

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29

128.7kD Sec31B-F

53kD Sec31B-T

Clone 184 85.2kD

53kD Sec31B-T

Clone 196 52.1kD

9.3kD 25.6kD Clone 205 77.9kD

![Diagram B](image)

**B**

![Diagram C](image)

**C**

Start 1 2 3 4 5 6 7 8 9 10 11 12 13

WD repeat >16% Proline

14 15 16 17 18 19 AB 20 21 22 23 24 25 26 27 28 29

>24% Proline

Stop

Fig. 3. Human Sec31B generates multiple transcripts by alternative mRNA splicing. Three cDNA libraries were examined for alternate exon utilization by PCR. Representative PCR results are shown in the supplementary material Fig. S1. (A) Alternative splicing map of Sec31B representing experimentally observed patterns of exon utilization, based on PCR analysis and EST data available in GenBank. Constitutive exons (green) and exons alternatively utilized (red) are shown along with the frequency of their detection in the examined transcripts. Of the total of 29 exons identified, 16 exons were constitutively present in all transcripts. (B) Examples of four alternatively spliced transcripts depicting their exon utilization. Arrows represent the open reading frames with the predicted molecular mass of the protein products. Although an open reading frame downstream of the stop codon in exon 13 exists, no evidence was identified for the utilization of initiation sites other than that in exon 2. Notice that, if exon 4 is not utilized, then exon 5 contributes an out-of-frame stop codon. (C) Cartoon representing all patterns of exon utilization experimentally identified in this study. Exons encoding WD repeats and proline-rich sequences are indicated.
immunofluorescent microscopy. Northern blots identify the predominant Sec31B mRNA as a series of two or three closely spaced bands centered at 6.5 Kb in all tissues examined, but most abundantly expressed in testis. Faint bands can also be noticed at approximately 1.0 kb and over 11 kb, the later presumably representing traces of genomic contamination. Analysis by dot-blotting of a multiple tissue array confirms abundant expression in testis as well as in cerebellum, thymus, lymph node, pituitary gland and, to a lesser extent, in all tissues on the array. A complete key to the dot-blot array is presented in supplementary material Table S1. (B) Western blots of a series of cultured cell lines using the rabbit IgG 2013 antibody, compared with the same blots probed with the antibody against Sec31A. Preimmune sera shows only a prominent non-specific band at approximately 80 kDa. This non-specific band is barely detectable with the affinity-purified IgG 2013, it demonstrates reactivity with Sec31B-F at 129 kDa as well as with a number of smaller bands. Notice that there is no correspondence of the bands reacting with IgG 2013 and the Sec31A antibody. (C) Cell- and tissue-extracts prepared from rat brain and testis were western blotted with anti-Sec31B antibodies Mab 1G10 and IgY 1871. The control lanes (ctrl) depict the reactivity present in non-immune hybridoma medium against MDCK cells (for Mab 1G10) or in the pre-immune IgY (for IgY 1871). Notice the presence of a 129 kDa band with both antibodies (Sec31B-F) and that only Mab 1G10 reacts with a prominent 53 kDa band (Sec31B-T). The star marks the position of the faint non-specific band just below the band for Sec31B-T.
translation initiation in Sec31B is even greater than what we have so far detected.

The intracellular disposition of Sec31B in testis and cerebellum was compared to Sec31A and Sec13 using Mab 1G10 (Fig. 5). In testis, Sec31B was present in Sertoli’s and Leydig’s cells, in spermatogonia and, less so, in mature sperm. The intracellular distribution of Sec31B was finely granular and diffuse, with concentration over the Golgi region and also in larger, dense cytoplasmic aggregates. It overlapped the distribution of Sec31A and Sec13 strongly in the Golgi region, in the diffuse cytoplasmic pools (presumably representing ER), but not in the more dense cytoplasmic accumulations. In the cerebellum, Sec31B was expressed in all layers, most prominently in Purkinje and granular cells. In Purkinje cells, Sec31B was densely concentrated into ten to 20 discrete cytoplasmic bodies (presumably the Golgi network) and, although also diffusely present in the cytoplasm, was not displayed in the same abundance as the coarse cytoplasmic aggregates found in the testis. By comparison, Sec31A in Purkinje cells displayed a prominent diffuse granular cytoplasmic distribution, with concentrations coincident with the staining of Sec31B over putative Golgi networks.

**Full-length Sec31B associates with other components of the COPII coat**

The pattern seen in testis and cerebellum, wherein the distribution of Sec31A and Sec31B was partially but not completely coincident, was also apparent in cultured cells. Three cell lines were examined, NRK cells, MDCK cells, and COS-7 cells; all gave comparable results. The pattern of Sec31B and Sec31A staining in COS-7 cells is shown in Fig. 6A. Overall, their staining was largely coincident, with concentration of both proteins over the Golgi network, as well as on dispersed small cytoplasmic puncta (Fig. 6A, top panel). However, they were not 100% coincident, with discrete and non-overlapping foci of Sec31B or Sec31A apparent. Presumably, the many peripheral small puncta that stained for both Sec31A and Sec31B represent ERESs. However, in some of the cultured cells the pattern of Sec31B was dominated by its collection into irregular coarse cytoplasmic aggregates, similar to the coarse accumulations observed in testicular tissue. Because we cannot reliably identify the distribution of just the truncated forms of endogenous Sec31B in these cells, we explored the behavior of transfected Sec31B-F and Sec31B-T, expressed as fusions with eYFP (or with eCFP analogues) in COS-7 or MDCK cells. These results are shown in Fig. 6A (lower panels). Whereas transfected eYFP-Sec31B-F consistently yielded a pattern indistinguishable from Sec31A (Fig. 6A, middle panel), transfected eYFP-Sec31B-T only partially overlapped the Sec31A staining (Fig. 6A, lower panel).

The previous results suggested that Sec31A and Sec31B-F shared the same intracellular distribution. Recent work has indicated that the Sec13-31 complex exists as a heterodimer. As a first step to determining whether Sec31B-F can form mixed dimers directly with Sec31A, lysates from a MDCK cell line stably expressing eYFP-Sec31B-F (MDCK-
Fig. 6. Sec31B-F and Sec31A share the same intracellular distribution. (A) The distribution of full-length and truncated Sec31B was compared with endogenous Sec31A in Cos-7 cells. In all panels, endogenous wild-type Sec31A is stained red; Sec31B (wild-type or transfected) is stained green. Areas of coincidence appear yellow. (Top) Comparison of the endogenous proteins; overlap is extensive, but not complete. (Middle) Comparison of wt Sec31A with transfected eGYP-Sec31B-F; overlap is essentially complete. (Bottom) Comparison of wt Sec31A with transfected eGYP-Sec31B-T; notice the absence of any overlap. Arrows indicate discrete non-overlapping accumulations of Sec31A and Sec31B. (B) Lysates of the MDCK-787 cell line that stably expresses eGYP-Sec31B-F were immunoprecipitated with the antibody against Sec31A (kindly supplied by Fred Gorlick, Yale University). The precipitates were then probed for the presence of either eGYP-Sec31B-F (left panel) or for Sec31A (right panel). Non-immunoprecipitated was used as the control (ctrl). Notice that anti-Sec31A does not cross-react with eGYP-Sec31B-F.

787) were immunoprecipitated with antibodies against Sec31A, and then western blotted to detect eGYP-Sec31B-F. These studies detected a clear and direct association between Sec31A and Sec31B-F (Fig. 6B).

The association of Sec31B with two other components of the COPII coat, Sec23 and Sec13, was also explored. Again, in native COS-7 cells, there was only partial overlap between Sec31B and Sec23 (Fig. 7A, top panel). As with the comparison with Sec31A, transfected eGYP-Sec31B-F demonstrated nearly complete overlap with Sec23, whereas transfected eGYP-Sec31B-T demonstrated no coincident staining. The close association of eGYP-Sec31B-F with Sec23 was further revealed by double-label electron microscopy (EM) (Fig. 7A). Both proteins were intermingled and coincident at the EM level, on both vesicular tubular clusters (VTCs) and on some 50-60-nm vesicles.

The association of Sec31B-F with Sec13 was detected by coincident staining in cultured cells, by communoprecipitation from native MDCK cells, and by GST-pull-down assays from MDCK cell lysates (Fig. 7B). The immunofluorescent results shown in Fig. 7 are from cells incubated at 15°C to block the release of COPII vesicles from the ERES. There is almost perfect coincidence of Sec31B-F with Sec13 staining at the putative ERES. Immunoprecipitation from the MDCK-787 line expressing eGYP-Sec31B-F with anti-GFP antibody co-precipitated Sec13. The region of Sec31B responsible for binding Sec13 was examined with GST-fusion peptides representing the N-terminal region of Sec31B with its WD repeats (residues 161 to 471), or a GST-fusion peptide (residues 818 to 1172) from the C-terminal region including its proline-rich domain. Only the peptide encompassing the WD repeats bound Sec13 in MDCK cell lysates (Fig. 7B).

Sec31B-F displays pharmacologic and dynamic behaviors that confirm its participation with COPII-coated organelles

To further characterize the association of Sec31B-F with the earliest steps in the secretory pathway, its distribution, response to blockage of ERESs or microtubule disruption, and its intracellular dynamics of motion were investigated. Incubation of cells at low temperature allows the accumulation of COPII-coated exit complexes along the ER, but prevents completion of their budding- and release-cycle. The MDCK-787 line was transfected with eCFP-VSV-G-tsO45, and the distribution of eCFP-Sec31B-F, eCFP-VSV-G-tsO45 and Sec13 were compared 4 minutes after the cells were returned to RT from a 15°C incubation. Under these conditions, a large number of ts045-VSV-G-CFP (VSV-G)-loaded and Sec13-positive VTCs are apparent. These are highlighted in Fig. 8A by the small arrows. Essentially, all of these clusters contain Sec31B-F. In an alternative approach, the same cell line expressing eCFP-VSV-
G-tsO45 was treated with nocodazole to disrupt their microtubules, under conditions in which the eCFP-VSV-G-tso45 could enter the secretory pathway after a brief incubation at a permissive temperature (Fig. 8B). Sec31B-F clumped into coarse punctate clusters dispersed throughout the cell, consistent with the behavior of other COPII components. The punctate clusters were loaded with VSV-G at permissive temperature.

Finally, the dynamic motion of Sec31B-F was examined and compared to the transport of tsO45-VSV-G-CFP in transfected COS-7 cells (Fig. 9). Whereas cargo laden vesicles exiting the ER undergo rapid long-range movement towards the Golgi network along microtubule pathways (Cole and Lippincott-Schwartz, 1995), COPII complexes typically remain tightly associated with the ER, and their movement is confined to within one or two microns of ERESs (Stephens et al., 2000). Consistent with its role as a COPII-coat component, eYFP-Sec31B-F was associated only briefly with VSV-G during its exit from the ER, and then remained relatively stationary as VSV-G moved towards the Golgi network.

Discussion
A novel mammalian orthologue of the yeast protein Sac31p has been cloned and characterized. This protein, termed Sec31B, arises from a 33 kb gene composed of 29 exons. Multiple isoforms of Sec31B are generated by alternative mRNA splicing and these isoforms fall into two distinct categories.

Fig. 7. Sec31B interacts with Sec13 and Sec23. (A) The distribution of full-length and truncated Sec31B was compared with endogenous Sec23 in COS-7 and MDCK cells. Fluorescent images from the COS-7 cells are shown. In all panels, endogenous wt Sec23 is stained red; Sec31B (wild-type or transfected) is stained green. Areas of coincidence appear yellow. (Top and middle rows) Comparison of the endogenous proteins or with transfected eYFP-Sec31B-F; overlap is extensive. (Bottom) Comparison of wild-type Sec23 with transfected eYFP-Sec31B-F; notice the absence of overlap. (EM) Thin sections of MDCK-787 cells embedded in LR white were stained with colloidal gold for Sec23 (5 nm particles) and for eYFP-Sec31B-F (10 nm particles). Notice the close association of staining over regions of vesicular tubular clusters (vtc) and on some 50-60 nm vesicles indicated by *. There is no staining of the Golgi cisternae (G). (B) Comparison of the distribution of endogenous Sec13 and eYFP-Sec31B-F in MDCK-787 cells that were also transfected with VSV-G, five minutes after incubation at permissive temperature (32°C). Staining of VSV-G is not shown, it was coincident with Sec13. Notice the perfect association of Sec13 staining with VSV-G in the punctate ERESs. (IP) Immunoprecipitation of the MDCK-787 cells with antibodies against GFP (Sec31B) or Sec13 co-precipitate Sec13. Non-immune sera (ctrl); cell lysates (lys). (GST pull-down) The direct interaction between Sec31B and Sec13 is mediated by the WD-repeat domain of Sec31B because a GST-fusion peptide encompassing the WD region of Sec31B efficiently binds Sec13 in MDCK cell lysates (WD), whereas a similar fusion peptide derived from the C-terminal proline-rich domain of Sec31B (PRD) that is devoid of the WD region does not bind Sec13. Ctrl, GST alone; lys, MDCK cell lysate used in the pull-down assay.
13 is not utilized, one – of several – full-length proteins (Sec31B-F) is translated that functions as a conventional COPII component. When exon 13 is utilized, its in-frame stop codon generates a family of truncated proteins (one which is Sec31B-T). These truncated proteins appear to associate with the ER but not in the same way as the full-length Sec31 proteins. These

**Fig. 8.** Sec31B-F moves with COPII complexes at different temperature or after disruption with nocodazole. The MDCK-787 cells expressing eYFP-Sec31B-F were transfected with VSV-G, and the dynamics of these proteins was measured under conditions of low-temperature blockage of ER export, or nocodazole disruption of microtubule-based intracellular transport. (A). Cells blocked at 15°C were warmed to room temperature (RT) for 4 minutes to allow entry of VSV-G into the ERESs. Cells were then fixed and examined for the above proteins. Under these conditions, VSV-G clusters into hundreds of minute ERESs that are coincident with COPII vesicular tubular clusters as detected by their Sec13 staining. The distribution of eYFP-Sec31B-F was perfectly coincident with these COPII-enriched ERESs. (B) Similar preparation of MDCK-787 cells blocked with nocodazole and incubated for 15 minutes at RT after a 2 hour incubation at 40°C. Under these conditions, eYFP-Sec31B-F forms coarse punctated clustes closely associated with the dispersed Golgi network to which VSV-G had moved during the 15 minute incubation at the permissive temperature (RT). Bars, 10 μm.

**Fig. 9.** VSV-G exiting the ER transiently associates with Sec31B-F. COS-7 cells were transiently transfected with VSV-G (construct 472, green) and eYFP-Sec31B-F (construct 787, yellow), colored red. After accumulating VSV-G in the ER for 3 hours at 40°C, cells were shifted to a permissive temperature (32°C) and images collected every 12 seconds over a period of 10 minutes. A selection of images at the times indicated is shown. The VSV-G can be seen filling the Golgi network. Also visible are many small transport vesicles exiting the ER and moving towards the Golgi network. The eYFP-Sec31B-F oscillates in the vicinity of ERESs but does not move towards the Golgi network. The insert, magnified 4x, demonstrates VSV-G and eYFP-Sec31B-F temporarily merged at the ERES; after 24 seconds the VSV-G vesicle moves anterograde towards the Golgi network, while eYFP-Sec31B-F stays behind. Arrows indicate the locus of a Sec31B-F vesicle (red) and a VSV-G-loaded vesicle (green). Bar, 10 μm.
conclusions are supported by several lines of evidence: (1) The Sec31B gene shows strong homology with both yeast Sec31p and mammalian Sec31A. (2) Multiple transcripts displaying alternative exon utilization have been generated by PCR from both brain and testis cDNA libraries. (3) Published ESTs confirm the cloning and PCR data indicating multiple alternative transcripts. (4) Western blotting with three independently generated and affinity-purified antibodies confirms both Sec31B-F and Sec31B-T transcripts in a variety of cell lines, and in testis extracts, as well as the presence of multiple immunoreactive bands consistent with many of the predicted alternative transcripts. (5) Brain and testis tissues and cultured cells display partially coincident staining for Sec31A, Sec13 and Sec31B. (6) Transfected GFP-tagged Sec31B-F and Sec31B-T do not exactly colocalize in cultured cells. (7) Sec31B-F binds Sec13 through its WD-repeat domains, and co-distributes with other COPII markers under all conditions examined. (8) Sec31B-F and Sec23 are coincident on 50-60 nm vesicles and VTCs by immunoemtomicroscopy. (9) Sec31B-F displays restricted intracellular movement, remaining in close proximity to putative ERESs whereas VSV-G moves rapidly away from these sites to the Golgi network, a dynamic identical to that of another COPII component, Sec23/24 (Stephens et al., 2000). Not addressed in this study is the role of the truncated isoforms of Sec31B, i.e. Sec31B-T and possibly other short isoforms arising from alternative translation initiation sites that are hinted at by some of the western blot data. Presumably, the presence of Sec31B-F bestows on a cell the capacity to generate a COPII coat with slightly modified dynamics or cargo specificities. It also appears probable that, based on the ability of Sec31B-F to coimmunoprecipitate with Sec31A (as well as with Sec13) and given recent indications that the functional Sec13/31 complex is a heterotetramer (Lederkremer et al., 2001), COPII-coat structures must exist, which not only contain various transcripts of Sec31B-F but also mixed heterotetramers of Sec13-Sec31B and Sec13-Sec31A. These results thus raise for the first time the intriguing possibility that the Sec13-Sec31 cage, like Sec23-Sec24, is built with isoforms tuned for specific types of cargo. Examples of such might include those reported for procollagen (Mironov et al., 2003), chylomicrons (Siddiqi et al., 2003) or GPI-linked proteins (Muniz et al., 2001). In future work, it will be important to explore not only the potential role(s) of the truncated forms of Sec31B, but also whether the efficient export of different cargos requires different combinations of Sec31B isoforms and Sec31A. If so, such a finding might explain the differential appearance of Sec31B seen in different tissues.

Materials and Methods
Cloning and sequencing
All molecular biological procedures followed standard methods (Maniatis et al., 1982) unless otherwise stated. Candidate sequences were derived from GenBank by TBLASTN searches using sequences from rat Sec31A (GenBank accession number AF034582). Oligonucleotides were designed to amplify the entire Sec31B cDNA from Clontech TH human testis, brain or fetal brain Marathon Ready cDNA libraries, using Platinum Taq high fidelity polymerase from Invitrogen Corporation, following manufacturer’s protocols. Primary and nested PCR, 5’ RACE, TA-TOPO cloning and sequencing were conducted as before (Stabach and Morrow, 2000). The oligonucleotides used in this study are listed in supplemental Figure S1. To eliminate errors associated with amplification, multiple overlapping PCR reaction products were cloned and sequenced over the entire length of Sec31B and compared, to the dbESTs from GenBank and the sequence of chromosome 10 (clone RP11-41I16) deposited from the Sanger Centre. Sequence analysis was performed using software from Gene Construction Kit, Texico Inc., and a portfolio of analysis tools from DNASTar Inc. The intron-exon boundaries were established by comparing the entire cDNA sequence against the sequence of chromosome 10, followed by heuristic inspection of the resulting pair-wise comparison for canonical splice junctions.

Northern analysis
Using a gel-purified fragment (bp 1331-2579) random labeled with [32P]dATP, a human normal mRNA blot IV (Invitrogen; catalog number D8104-08) was probed following the manufacturer’s instructions. The blot was then stripped and probed for actin transcripts to confirm equal loading of housekeeping genes. Similarly, a human Sec23 probe was used with a Multiple Tissue Express Array (Catalog number 7775-1, Clontech, Palo Alto) to examine a wider tissue distribution.

Production of prokaryotic fusion proteins and eukaryotic expression vectors
Cloning of the N-terminal region of Sec31B containing the WD motif was accomplished by PCR using oligonucleotides 51853 and 51865 (supplementary material Fig. S1). PCR products were gel-purified from low-melt agarose (FMCD) using Pharmacia GEL GEX gel purification kit, digested with EcoRI and cloned into pGEX-4TI (Pharmacia). Expression of fusion protein occurred in DH5a bacteria after the addition of 0.1 mM IPTG. Cloning of the C-terminal proline-rich domain was accomplished in a similar way using oligonucleotides 48827 and 49372, except that the PCR product was digested with both EcoRI and BamHI to take advantage of the internal BamHI site located within the amber. Full-length Sec31B-F was assembled into the Xhol and AccI sites of pEGFP-N3 (BD Biosciences) using sequenase-verified PCR amplimers. During this ligation the AccI site was destroyed after annealing with the compatible overhang of a Clal site, part of sense oligo 63730 used to amplify Sec31B. This construct was then digested with Smal and DrelIII and the GFP part of the vector replaced with a yellow fluorescent protein from pEYP-1N (BD Biosciences). The truncated version Sec31B-T was constructed in a similar way by ligating a PCR amplifier, which contained the endogeneous stop codon from exon 13, into the Xhol and Kzm sites of pEYP-CN (BD Biosciences).

Creation of the stable MDCK-787 cell line expressing eYFP-Sec31B-F
The cDNA for eYFP-Sec31B-F (787) was transfected into MDCK cells using Lipofectamine 2000 following the manufacturer’s instructions. After 48 hours the cells were plated at dilutions into Dulbecco’s minimal Eagle’s medium (DMEM) supplemented with 400 μg/ml of G418 (Geneticin, Invitrogen). After three weeks, G418-resistant colonies were subcloned and screened by fluorescent microscopy for the expression of eYFP. Positive clones were expanded and tested by western blot using an anti-GFP antibody for the expression of a single 158 kDa band. Four independent colonies exhibiting the best expression data were chosen for use in these studies. All gave identical results.

Antibody preparation and immunologic procedures
Three antibodies were prepared for this study. An IgY antibody (IgY1871) (Avex labs, Davis, OR) against a recombinant peptide generated by expressing cDNA corresponding to residues 818-1172, exons 16 to 29, in pGEX-3T (Amersham Pharmacia Biotech) was prepared in chickens. This antibody was affinity-purified by desorption from Millipore Immobilon-P membranes loaded with the GST-tagged Sec31B peptide. Affinity purified antibodies were eluted from Immobilon-P membranes with 200 mM glycine, 500 mM NaCl pH 2.8, and neutralized with 1 M Tris pH 8.0. The second antibody prepared was a mouse monoclonal antibody (Mab). Two independent Mab clones (Mab 1D4 and 1G10) (Maine Biotechnologies) were identified against a GST-fusion peptide derived from exons 5-13 of Sec31B. The third antibody was an affinity-purified rabbit IgG (IgG2013) against a synthetic peptide bridging exons 16 and 17 of Sec31B. The peptide sequence MTPWEITPKIDGIL corresponds to codons 552-566. We chose this peptide because it shares little homology to Sec31A or Sec31p. All antibodies were evaluated for specificity by: (i) comparison with preimmune sera; (ii) by competitive inhibition of western blots by the recombinant or synthetic peptide used to generate the antibody; and (iii) by confirmation of specificity using western blots of cell extracts overexpressing the recombinant Sec31B peptides as eYFP-fusion proteins. The antibody directed against GFP was from Clontech, the Sec23 antibody was a rabbit IgG from Affinity Bioreagents. Other antibodies were obtained from private sources as indicated in the acknowledgements.

Immunocytochemistry
COS-7 and MDCK cells were grown in Eagle’s or DMEM with 10% fetal BSA on Falcon tissue culture chamber slides. Both wild type cells and cells transfected by Lipofectamine 2000 (Invitrogen) were fixed with either 50:50 v/v mixture of acetone/methanol at –20°C for 10 minutes, permeabilized with 0.1% saponin in PBS; and incubated with Mabs 1G10 or 1G10 diluted 1:50 in PBS with 0.1% saponin, 2% BSA (Fraction V; Sigma Chemical Co., St Louis, MO). Cells were washed with PBS, and incubated with 1:1000 secondary antibody conjugated to Alexa Fluor 488 or Alexa Fluor 568 (Molecular Probes).

968 Journal of Cell Science 119 (5)
Electron microscopy

Cells grown on 10 cm plastic Petri dishes were fixed overnight with a mixture of 4% formaldehyde and 0.1% glutaraldehyde in PBS pH 7.4. Cells were collected and pelleted, and cell pellets were washed with PBS, dehydrated on ice in graded ethanol before LR White resin-infiltration. Resin was polymerized in sealed gelatin capsules at 30 °C for 48 hours. Thin sections were cut and adhered to carbon-coated formvar grids. Gold labeling was performed with anti-GFP (Clontech), and anti Sec-23 (ABR) antibodies. Thin sections were blocked with 1% BSA, 0.1% glycine in PBS, incubated with Sec23 antibody for 2 hours followed by 1 hour incubation with 5 nm goat anti-rabbit colloidal gold. Cells were re-fixed with 1% glutaraldehyde, re-blocked and labeled with GFP antibody followed by 10 nm goat anti-rabbit colloidal gold. Sections were then re-fixed in 1% glutaraldehyde, PBS washed, air-dried and post stained with 0.5% uranyl acetate and 1 mg/ml lead citrate. Sections were examined and imaged on a Zeiss EM-910 microscope at 80 KV.

tsv545-VSV-G-eCFP (VSV-G)-transport studies

The tsv545-VSV-G-eCFP (VSV-G) construct was generated from a tsv545-VSV-G-GFP clone (a gift from J. Lippincott-Schwartz, NIH) by replacement of the GFP with the enhanced cyan fluorescent protein (eCFP) derived from pECFP-1N (BD Biosciences). COS-7 cells transiently co-transfected with full-length eYFP-Sec31B (or with truncated variants) and VSV-G, were incubated at the non-permissive temperature of 40 °C for 3-6 hours to load the ER with newly translated VSV-G, and then shifted to either 15 °C (to block exit at the ERES) or 32 °C to study the kinetics of synchronized transport of VSV-G to the Golgi network. Fluorescence microscopy was performed on the Olympus BX51 digital camera, Olympus LEXTO dual laser scanning confocal microscope, or with an Olympus AX70 equipped with OpenLab™ (Improvison, Lexington, MA) deconvolution software. Time-lapse vital cell microscopy was done with an Olympus IX70 dual laser scanning confocal microscope, all controlled by TILL vision software (TILL Photonics LLC, Pleasanton, CA).

Other procedures

The interaction of the recombinant Sec31B peptides with Sec13 was determined by the ability of GST-Sec31B peptides to capture Sec13 from MDCK cell extracts in pull-down assays or by communoprecipitation. MDCK cells were extracted with a non-denaturing buffer (20 mM Hepes pH 7.4, 120 mM NaCl, 25 mM KCl, 2 mM EDTA, 1 mM EGTA, 0.1% Triton X-100). Typically, 0.1 mg of GST-Sec31B peptide in 50 mL Tris pH 8.0, 50 mM NaCl, 1 mM EDTA, 1 mM DTT would be absorbed at 50 μl of 1:1 slurry of glutathione-Sepharose. Proteins retained after three washes in the same buffer were analyzed by western blot after SDS-PAGE. For immunoprecipitation experiments, MDCK cell extracts were pre-cleared with Protein A-tris acryl (Pierce), incubated overnight at 4 °C with primary antibodies and proteins captured with Protein A-tris acryl. Proteins retained on the resin after three washes were analyzed by western blot.

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