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Translocon-associated protein TRAPδ and a novel TRAP-like protein are coordinately expressed with pro-opiomelanocortin in *Xenopus* intermediate pituitary

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In the intermediate pituitary gland of *Xenopus laevis*, the expression levels of the prohormone pro-opiomelanocortin (POMC) can be readily manipulated. When the animal is placed on a black background, the gene for POMC is actively transcribed, whereas on a white background the gene is virtually inactive. In this study, we characterized two genes whose transcript levels in the intermediate pituitary are regulated in coordination with that for POMC. One of these codes for a protein homologous to translocon-associated protein TRAPδ, a subunit of a transmembrane protein complex located at the site where nascent secretory proteins enter the endoplasmic reticulum (ER). Both *Xenopus* and mice were found to express an alternatively spliced transcript that gives rise to a previously unknown version of the TRAPδ protein. The product of the second gene is a novel and highly conserved protein with structural similarity to glycoprotein gp25L, a constituent of another translocon-associated protein complex. A database search revealed the existence of a novel family of gp25L-related proteins whose members occur throughout the animal kingdom. Together, our data imply that (i) the group of ER proteins surrounding translocating polypeptide chains may be far more complex than previously expected, and (ii) a number of the accessory components of the translocon participate in early steps of prohormone biosynthesis.

INTRODUCTION

The endoplasmic reticulum (ER) forms the entrance of a biosynthetic transport route by which a complex mixture of both secretory and transmembrane proteins reach their final destinations. Virtually all proteins that require transport to the cell surface, lysosomes or storage vesicles, as well as those residing in the ER or Golgi complex, first undergo translocation across or insertion into the ER membrane. These events take place at specific sites (translocons) and involve hydrophilic protein-conducting channels whose major components were initially identified in yeast by genetic screening for translocation defects [1,2]. Experiments in which the mammalian translocation process was reconstituted from purified components have indicated that transport of polypeptides essentially requires only two integral membrane–protein complexes, namely the signal recognition particle receptor, which is responsible for targeting the nascent polypeptide chain to the ER, and the Sec61p complex, which is believed to form the actual protein-conducting channel [3]. The translocation of a subset of polypeptides also depends on the presence of a third membrane component, the translocating chain-associating membrane (TRAM) protein [3,4]. By means of (photo)chemical cross-linking techniques, several additional membrane proteins have been identified that are in close molecular proximity to translocating nascent chains. Among these is the translocon-associated protein TRAPα (previously the signal sequence receptor SSRα), a type I transmembrane glycoprotein that interacts with both nascent secretory as well as membrane protein precursors during translocation [5,6]. TRAPα can be cross-linked to membrane-bound ribosomes [7], and Fab fragments of antibodies raised against the protein were found to inhibit the translocation of several secretory proteins *in vitro* [8]. However, immunodepletion of TRAPα from detergent extracts of rough microsomes had no effect on translocation activity [9]. It therefore appears that TRAPα, although representing another constituent of the translocon, is not required in the translocation process itself. At present, the function of TRAPα is unknown. Interestingly, the protein was found to be tightly and stoicheiometrically associated with a second single-spanning membrane glycoprotein, named TRAPβ (previously SSRβ) [10]. Wada et al. [11] have isolated a hetero-tetrameric protein complex in which TRAPα and TRAPβ are associated with calnexin and a small transmembrane glycoprotein, gp25L. In contrast, Migliaccio et al. [9] and Hartmann et al. [12] purified another complex in which the TRAPα–β dimer is associated with a type IV transmembrane protein, TRAPγ, and a single membrane-spanning protein, TRAPδ. It is possible that differences in the purification procedures employed by these research groups have led to the isolation of distinct protein complexes. Alternatively, the variability observed in these protein complexes may reflect different substrate specificities of the translocon apparatus or be related to discrete steps in the translocation event.

Our research interest is focused on the molecular mechanisms involved in the biosynthesis of peptide hormones and their stimulus-dependent release from neuroendocrine cells. We recently developed a strategy for the selective cloning of components from the secretory pathway in *Xenopus* melanotropes, taking advantage of the fact that the biosynthetic and secretory activity of these intermediate pituitary cells can be readily modulated *in vivo* (J. Holthuis, E. Jansen, M. van Riel and G. Martens, unpublished work). When the animal is placed on a black background, the melanotropes produce and release high quantities of pro-opiomelanocortin (POMC)-derived, melanophore-stimulating peptides, which cause a darkening of the skin [13]. In contrast, melanotrope cells of animals on a white background are relatively inactive, resulting in a light skin. Consequently, the melanotropes of black-adapted animals contain up to 30-fold more POMC mRNA than those of white-

Abbreviations used: ER, endoplasmic reticulum; EST, expressed sequence tag; NIL, neurointermediate lobe; ORF, open reading frame; POMC, pro-opiomelanocortin; RT–PCR, reverse-transcription PCR; ss, single-stranded; TRAP, translocon-associated protein.

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adapted animals [14]. We differentially screened an intermediate pituitary cDNA library with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals. We here describe the characterization of two genes whose transcript levels are regulated in coordination with that of POMC. One of these codes for the *Xenopus* homologue of TRAPδ whereas the other gives rise to a member of a novel family of gp25L-related proteins.

MATERIALS AND METHODS

Animals

South African clawed toads *Xenopus laevis* were bred and reared in the aquarium facility of the Department of Animal Physiology at the University of Nijmegen. Animals (40–60 g) were adapted to black or white backgrounds under constant illumination for 3 weeks at 22 °C.

cDNA library screenings

Cytoplasmic RNA was isolated from neurointermediate lobes (NILs) of 100 black-adapted *Xenopus* toads by using the Nonidet P40 method and subjected to oligo(dT) chromatography according to Sambrook et al. [15]. cDNA appropriate for directional cloning was synthesized with a commercial cDNA synthesis kit (Stratagene), size-fractionated on CL4B Sepharose and ligated into λ uni-ZAP XR (Stratagene). About 50000 primary plaques were hybridized with a random-prime-labelled POMC cDNA fragment and a single-stranded (ss) cDNA probe synthesized with Superscript reverse transcriptase (Gibco-BRL) from oligo(dT)-primed *Xenopus* liver RNA, by using standard procedures [15]. Recombinant pBluescript SK$^-$ phagemids were excised *in vivo* from 204 non-hybridizing λ ZAP clones, rescued as ss antisense DNA and spotted on to a duplicate set of nitrocellulose filters. As a prerequisite for the generation of probes for the differential screening, total NIL RNA of six black-adapted and six white-adapted animals was used for the synthesis of oligo(dT) cellulose-coupled cDNA according to the method of Rodriguez and Chader [16]. Ss-sense cDNA probes (the black and white probes) were then prepared by random prime-labelling of each cellulose-coupled cDNA. Filters were hybridized separately overnight at 63 °C with the black probe (total radioactivity 4×10^6 c.p.m.) and white probe (total radioactivity 0.5×10^6 c.p.m.), washed to a final stringency of 0.2 × SSC (where SSC is 0.15 M NaCl/0.015 M sodium citrate) at 63 °C and exposed to X-ray film for 3 days at −70 °C with two intensifying screens. Next, cDNA inserts from two selected clones, X0286 and X1262, were random-prime-labelled and used to rehybridize the primary *Xenopus* NIL cDNA library. Since this procedure yielded a full-length cDNA for X0286 only, 1.2×10^6 plaques from an amplified λ ZAP-II *Xenopus* hypothalamus cDNA library [17] were hybridized to obtain the full-length X1262 cDNA clone. About 6×10^5 plaques from an amplified human fetal brain λ ZAP II cDNA library (Stratagene) and 8×10^5 plaques from an amplified mouse λ uni-ZAP XR brain cDNA library (Stratagene) were screened for X0286 cDNA homologues. Hybridization of the human and mouse libraries was performed overnight in hybridization buffer (6 × SSC, 0.5% SDS, 0.1% pyrophosphate, 40 mM sodium phosphate, pH 7.0, 1 mM EDTA, 3 × Denhardt's and 0.1 mg/ml denatured salmon sperm DNA) at 42 °C and filters were washed to a final stringency of 0.4 × SSC at 60 °C.

DNA sequence analysis and database matching

Sequencing of selected cDNA clones on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA by using T7 DNA polymerase (Pharmacia) and the dideoxy chain-termination method [18]. Nucleotide sequences and deduced protein sequences were compared with those present in the EMBL/Genbank and SwissProt/PIR databases using the computer facilities of the CAOS/CAMM centre at the University of Nijmegen.

RNA isolation

For expression studies, total RNA was prepared according to the method of Chomczynski and Sacchi [19], using acid/guanidine isothiocyanate/phenol/chloroform extraction. After recovery by ethanol precipitation, the RNA was quantified by spectrophotometry and its integrity checked by running samples on denaturing agarose gels followed by ethidium bromide staining. Poly(A)$^+$ RNA was selected by oligo(dT) chromatography and quantified by spectrophotometry. For the recovery of RNA from *Xenopus* neurointermediate and anterior lobes, yeast tRNA was used as a carrier.

RNase protection assay

Constructs to be used in the RNase protection assay were generated by subcloning appropriate restriction fragments from selected cDNA clones into pBluescript SK$^-$ vector. After verification by sequencing, ~100 ng of linearized construct was used for the generation of antisense run-off transcripts from the T3 or T7 RNA polymerase promotor. Transcripts labelled with [α-^{32}P]UTP (800 Ci/mmol, Amersham) were purified on 5% (w/v) polyacrylamide/8 M urea gels. Sizes of transcripts generated from the constructs (with sizes of protected bands in parentheses) were: X0286, 402 nt (346); X1262, 216 nt (156); POMC, 301 nt (271); fascin, 406 nt (311). About 10^5 c.p.m. of each transcript was combined with total RNA samples in 25 μl of hybridization mix [80% (v/v) formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, containing 1 mM EDTA]. Samples were incubated at 80 °C for 5 min before hybridization overnight at 50 °C. Non-hybridized RNA was digested with RNase A and RNase T1 for 30 min at 37 °C. Samples were treated with proteinase K, extracted with phenol/chloroform/isoamyl alcohol, supplemented with 10 μg of yeast tRNA, precipitated with ethanol and run on a 5% (w/v) polyacrylamide/8 M urea gel. After autoradiography, quantification of protection signals was performed with an Ultrascan XL laser densitometer (LKB/Pharmacia).

Northern blot analysis

Poly(A)$^+$ RNA from *Xenopus* liver, brain, heart and kidney was separated by electrophoresis on 2.2 M formaldehyde-containing 1.2% (w/v) agarose gels in Mops buffer and blotted onto nitrocellulose filters as described by Ausubel et al. [20]. Hybridization was overnight at 45 °C in 5 × SSPE [where SSPE is 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 50% (v/v) formamide, 5 × Denhardt's, 0.5% SDS and 0.1 mg/ml denatured salmon-sperm DNA. Probes (10^6 c.p.m. per ml of hybridization buffer) were prepared by random prime-labelling of cDNA inserts from clones X0286 and X1262. Blots were washed at 63 °C to a final stringency of 0.1 × SSPE and autoradiographed for 3 days at −70 °C with two intensifying screens.

Reverse transcription–PCR analysis

Total RNA extracted from various tissues was annealed to oligo(dT) primer, and ss cDNA was synthesized with Superscript

reverse transcriptase (RT). The cDNA was amplified with 50 pmol each of primer 1 (5′-CAGAAGTGGTTTTCATCGTGGAGAT-3′) and primer 2 (5′-TTAAGCCTGGATGTTGCTCTTTGC-3′) for 30 cycles (93 °C, 1 min; 60 °C, 1.5 min; 70 °C, 1 min; Perkin Elmer-Cetus Thermal Cycler) with Super-*Taq* DNA polymerase (Perkin Elmer-Cetus). Primer 1 corresponds to nucleotides 179–203 and primer 2 is the reverse complement of nucleotides 544–567 in cDNA clone X0286 (see Figure 3). PCR products were run on a 1.5% agarose gel, blotted onto nitrocellulose and hybridized under standard conditions [15] with random-prime-labelled X0286 cDNA insert as a probe. Autoradiography was for 3 h at room temperature.

RESULTS

Isolation and characterization of mRNAs co-ordinately expressed with POMC in *Xenopus* intermediate pituitary during background adaptation

As an approach towards the identification of genes involved in peptide hormone biosynthesis and release, we have screened an intermediate pituitary cDNA library of black-background-adapted *Xenopus* with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals. This screening yielded 37 differentially hybridizing clones corresponding to a total of 14 distinct transcripts whose levels in the melanotropes are regulated in coordination with that of POMC (J. Holthuis, E. Jansen, M. van Riel and G. Martens, unpublished work). Multiple independent cDNAs were isolated for transcripts encoding prohormone processing enzymes (carboxypeptidase H and prohormone convertase PC2) and members of the granin family of proteins (secretogranins SGII, SGIII and the neuroendocrine polypeptide 7B2). Because these proteins are all packaged and released together with peptide hormones, their co-expression with POMC in the melanotropes was not unexpected. Of the remaining transcripts, those represented by clones X0286 and X1262 displayed the strongest alterations in expression levels. RNase protection experiments indicated that NILs of black-adapted animals contain 20-fold more X0286 transcripts and 15-fold more X1262 transcripts than those of white-adapted animals (Figure 1). These magnitudes of regulation are similar to that observed for POMC mRNA, whose level is altered 20–30-fold during background adaptation [14]. As previously demonstrated for POMC, the physiologically induced changes in X0286 and X1262 transcript levels are confined to the melanotropes of the NIL and do not occur in cells from the anterior lobe of the pituitary (Figure 1). Such an expression pattern is restricted to a limited set of genes, excluding for instance those encoding ferritin, actin or the actin-bundling protein fascin [21] (Figure 1, and results not shown). Northern blot analysis showed that clone X0286 represents an mRNA of 0.8 kb that is expressed in *Xenopus* liver, brain, heart, kidney and lung (Figure 2). Clone X1262 corresponds to a 1.2 kb transcript with an expression

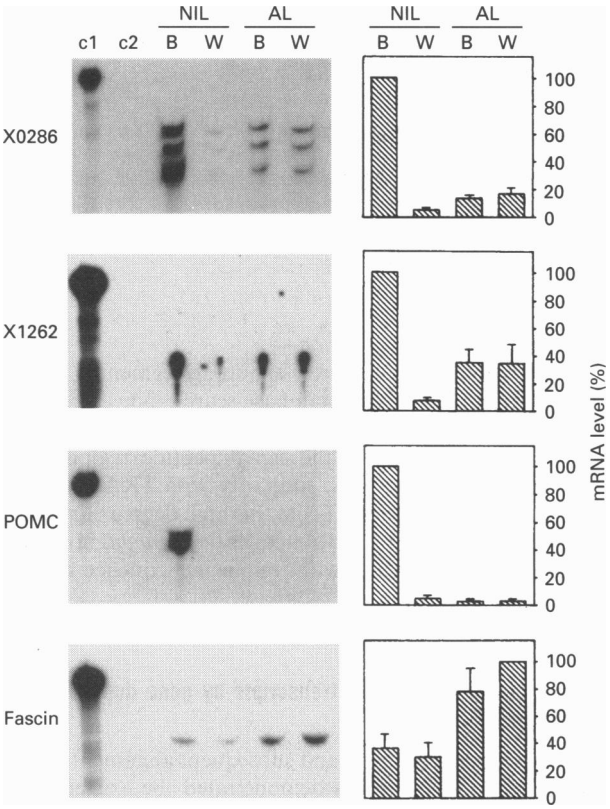

Figure 1 RNase protection analysis of X0286, X1262 and POMC transcripts in the *Xenopus* pituitary

Radiolabelled anti-sense RNA was hybridized to total RNA extracted from NILs and anterior lobes (AL) of black- (B) or white-adapted (W) *Xenopus*. In each experiment, total RNA from two lobes was used, except for POMC (1/25 part of each lobe). Samples were digested with RNase A and RNase T1, loaded onto denaturing polyacrylamide gels and autoradiographed. An anti-sense probe against the non-regulated fascin mRNA served as a control. The multiple protection signals observed for X0286 probably relate to the divergence observed in the nucleotide sequences of two related transcripts (see Figure 2 and text). Transcript levels were quantified by densitometric scanning of autoradiographs. Data shown are the means ± S.D. of three independent experiments. c1, undigested RNA probe; c2, RNA probe hybridized to 20 μg of yeast tRNA before digestion.

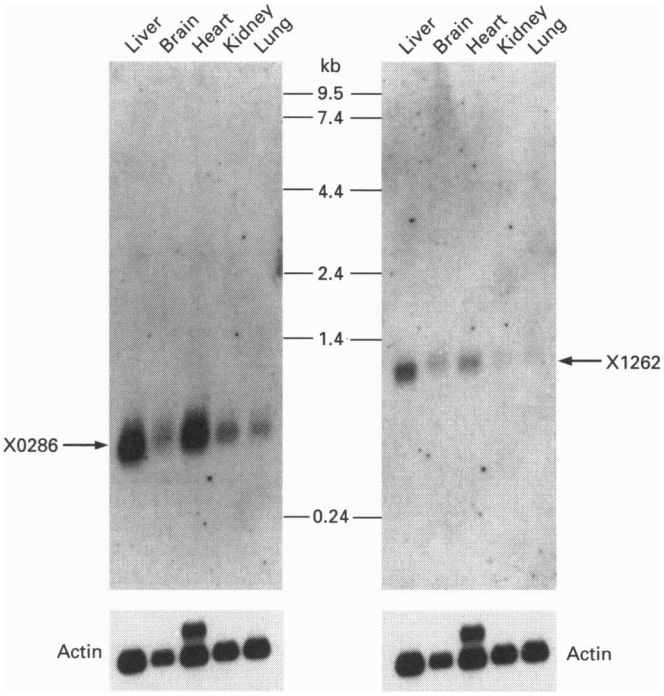

Figure 2 Northern blot analysis of X0286 and X1262 transcripts

Random-prime-labelled inserts from cDNA clones X0286 and X1262 were hybridized to 7.5 μg of poly(A)$^+$ RNA from *Xenopus* liver, brain, heart, kidney and lung. As a control for loading, blots were stripped and rehybridized with an actin cDNA probe. The mobilities of RNA size markers are indicated.

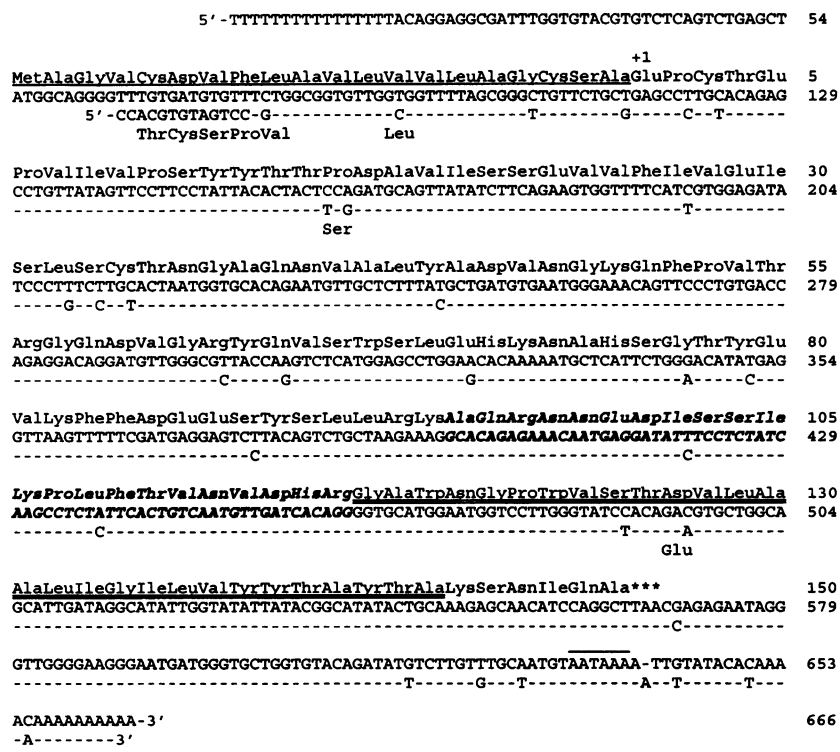

Figure 3 Nucleotide sequence and deduced amino acid sequence of *Xenopus* intermediate pituitary cDNA clone X0286

Substitutions in the sequence of a closely related cDNA are indicated. The signal peptide sequence is underlined. Positive numbering of amino acids starts in the mature protein. Doubly underlined amino acids are predicted to span the membrane. The nucleotide sequence and deduced amino acid sequence shown in bold italics are deleted in an alternatively spliced form of the X0286 transcript. Overlined is the consensus for polyadenylylation.

profile similar to that of the X0286 mRNA, yet with lower levels of expression (Figure 2). RNase protection experiments demonstrated that both transcripts are also present in muscle, gut, pancreas, spleen, adipose tissue, testis, ovaria and oocytes (results not shown). Together, these results indicate that the expression of X0286 and X1262 is not limited to specific organs or cell types but that both transcripts are derived from ubiquitously expressed genes.

Transcript X0286 encodes the *Xenopus* homologue of TRAPδ

Clone X0286 contained a cDNA insert of 0.6 kb. Nucleotide sequence analysis revealed an open reading frame (ORF) of 167 amino acids lacking a translation initiation codon. Rescreening of the intermediate pituitary cDNA library gave five positive clones with insert sizes ranging from 0.5 to nearly 0.7 kb. Sequence analysis of the largest insert revealed a 54 bp 5′-untranslated region, a 510 bp ORF and a 102 bp 3′-untranslated region with a putative polyadenylation signal 17 bp upstream of the poly(A) tract (Figure 3). A potential translation initiation site (5′-TGAGCT*ATG*G-3′, where the start codon is italic) [22] is present at nucleotide positions 49–58. Translation from this site would generate a polypeptide of 170 amino acids with a predicted molecular mass of 18574 Da. The initiator methionine precedes a predominantly hydrophobic region of 20 amino acids, strongly resembling a signal sequence with a cleavage site conforming to the -1, -3 rule [23]. Removal of this region would leave a polypeptide of 150 amino acids with a molecular mass of 16654 Da. A potential membrane-spanning segment is found at residue positions 117–144. A database search indicated that the X0286 sequence is closely related to that of TRAPδ from the rat (Figure 4). When excluding the signal peptide region, X0286 shares 80% identity and 90% similarity with TRAPδ over 150 matched amino acid residues. From this high degree of structural identity we conclude that X0286 encodes the *Xenopus* homologue of TRAPδ. No other entries with significant sequence similarity to X0286 were present in the database.

Generation of multiple TRAPδ transcripts by gene duplication and alternative mRNA splicing

Nucleotide sequence analysis and subsequent alignment of all six isolated X0286 cDNA clones demonstrated the existence of at least three distinct transcripts for TRAPδ in *Xenopus*. First, we noticed 43 substitutions over 602 matched nucleotides (7%) between the original X0286 clone and the five clones from the rescreening (Figure 3). These substitutions caused considerable alterations in the signal peptide sequences, whereas structural changes in the mature protein are limited to two conservative substitutions: $Pro^{16} \rightarrow$ Ser and $Asp^{127} \rightarrow$ Glu. It therefore seems that two structurally distinct TRAPδ proteins are expressed from a closely related pair of genes. In *Xenopus*, several other gene pairs have been described whose exon sequences diverge by between 4% and 9%. Included are the genes for vitellogenins [24], albumins [25], proenkephalins [26], L1 ribosomal proteins [27] and POMCs [28]. These gene pairs are believed to originate

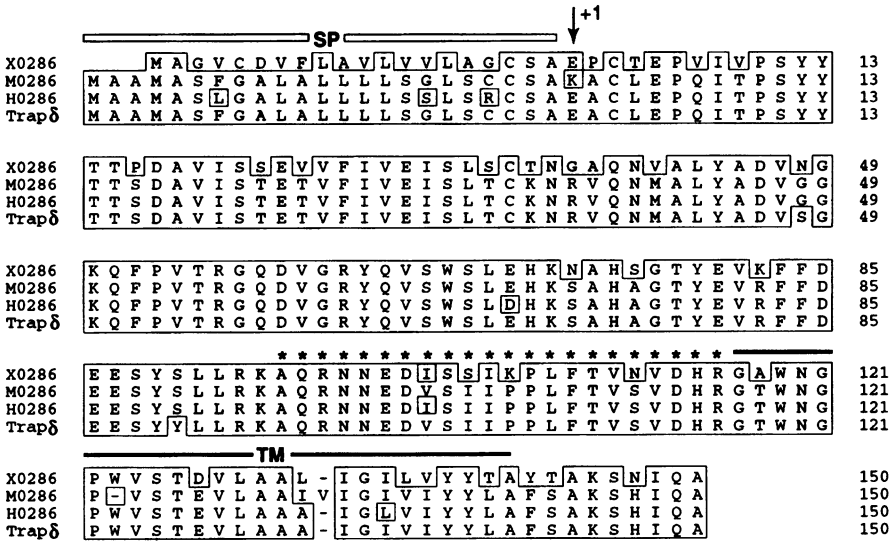

Figure 4 Alignment of amino acid sequences deduced from *Xenopus* cDNA X0286, mouse cDNA M0286, human cDNA H0286 and rat TRAPδ

The single-letter amino acid code is used. Gaps are introduced for optimal alignment. Signal peptide (SP) and transmembrane (TM) regions are overlined and the putative signal peptide cleavage site is indicated by an arrow. Residues deleted in alternatively spliced forms of the *Xenopus* and mouse mRNAs are starred. The rat TRAPδ sequence was taken from Hartmann et al. [12]. The cDNA sequences of X0286, M0286 and H0286 are available from the EMBL database under accession numbers X90584, X90582 and X90583 respectively.

from a duplication of the entire *Xenopus* genome that took place about 30 million years ago [29,30].

A second remarkable finding was that three of the six isolated X0286 cDNA clones showed a deletion of 66 nucleotides from position 397 to 462. Consequently, the proteins encoded by these cDNAs lack a stretch of 22 amino acid residues (from Ala^{95} to Arg^{116}) immediately N-terminal of the membrane-spanning segment (Figure 3). This finding suggests that the transcript of at least one of the two TRAPδ genes undergoes alternative splicing, thereby giving rise to two structurally distinct TRAPδ proteins of ~14 and ~17 kDa. RT–PCR was used to study the expression of alternatively spliced TRAPδ transcripts in a variety of *Xenopus* tissues (for details see the Materials and methods section). After Southern blot analysis, PCR products of approximately 325 bp and 400 bp, each corresponding to one of the alternatively spliced transcripts, were detected in pituitary (NIL and anterior lobe), pancreas, kidney, liver, heart, gut, spleen, muscle and skin (results not shown). Alternative splicing of the TRAPδ transcript therefore occurs in a wide variety of tissues and probably does not represent a cell-type-specific event.

To analyse further the conservation of TRAPδ during evolution and to find out whether alternative splicing of its transcript also occurs in mammals, we screened brain cDNA libraries from mouse and human, with radiolabelled X0286 cDNA as a probe. Six positive clones from the mouse library and two from the human library were isolated with cDNA inserts varying from 0.7 kb to nearly 1 kb in size. These clones each contained an ORF for a protein highly homologous to TRAPδ (Figure 4). The mouse and human proteins share an overall sequence identity of 80% with *Xenopus* TRAPδ, 97% identity with rat TRAPδ and 97% identity with each other. One of the six isolated mouse cDNA clones showed a deletion of 66 nucleotides within the ORF and thus the protein encoded by this cDNA lacks TRAPδ residues Ala^{95} to Arg^{116} (see Figure 4). This in-frame deletion is located at exactly the same position as that observed in some of the X0286 cDNAs. Hence alternative splicing of the TRAPδ transcript is a conserved phenomenon that takes place in both amphibians and mammals.

Transcript X1262 encodes a protein structurally related to mammalian glycoprotein gp25L and two yeast transmembrane proteins

Clone X1262 contained a cDNA insert of 1 kb with an ORF of 186 amino acids from which the start methionine is lacking. Rescreening of the melanotrope cDNA library did not yield clones with 5′-extended sequences. We therefore hybridized a *Xenopus* hypothalamus cDNA library using the X1262 insert as a probe. Six of the 21 positive clones were selected for sequence analysis. The largest of these contained a 1.1 kb cDNA comprising a 29-bp 5′-untranslated region, a 615-bp ORF and a 484-bp 3′-untranslated region with three polyadenylation signals upstream of the poly(A) tail (Figure 5). Translation from the start codon at nucleotide position 30 would produce a polypeptide of 23786 Da with an N-terminal 18-residue hydrophobic sequence reminiscent of a signal peptide. Use of the predicted peptide cleavage site would leave a 187-residue polypeptide with a calculated molecular mass of 21795 Da. A potential N-linked glycosylation site was found at residue position 147 and a stretch of hydrophobic residues, long enough to span a lipid bilayer, is located close to the C-terminus at residue positions 154–176. Alignment of the cDNA sequences from all six X1262 clones revealed that one of these contains nine substitutions over 1042 matched nucleotides (1%). Two amino acid substitutions were found in the ORF, namely $Pro^{48} \rightarrow$ His and $Tyr^{49} \rightarrow$ Ile (Figure 5). Hence, like TRAPδ, X1262 in *Xenopus* is generated by a closely related pair of transcriptionally active genes.

A database search indicated that the X1262 nucleotide sequence is 70% identical with two human expressed sequence tags (HS23814 and HBC1303) over a total length of 321 nucleotides. At the protein level, X1262 shares 85% sequence identity (91% similarity) over 100 matched amino acid residues with the

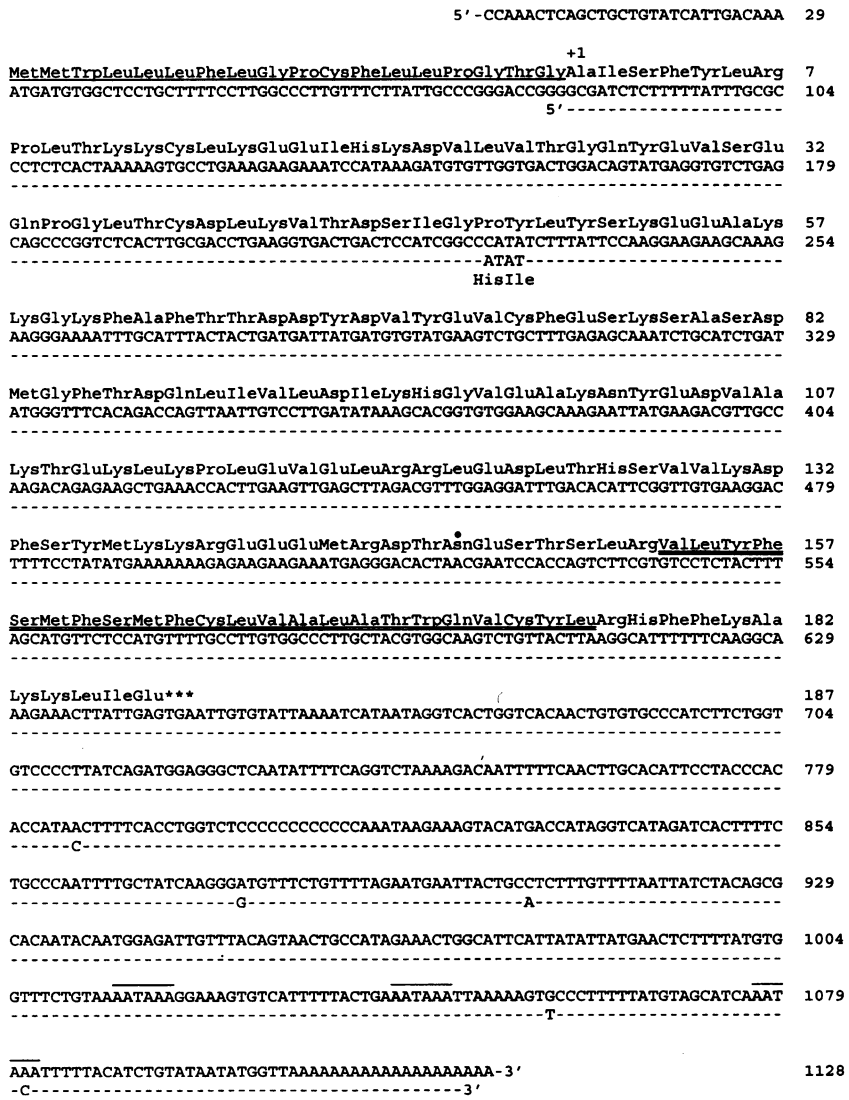

Figure 5 Nucleotide sequence and deduced amino acid sequence of *Xenopus* intermediate pituitary cDNA clone X1262

Substitutions in the sequence of a closely related cDNA are indicated. A potential Asn-linked glycosylation site is marked by a black dot. Other designations are as in Figure 3.

expressed sequence tag (EST)-derived polypeptide sequences (Figure 6, Table 1). The EST-encoded protein thus probably represents the human homologue of X1262. The database search revealed three additional entries showing low but significant degrees of similarity between the X1262 protein and dog glycoprotein gp25L, yeast hypothetical protein Yhr110w, and yeast protein p24B (Figure 6). In its mature form, the X1262 protein shares an overall sequence identity of 31% (52% similarity) with gp25L, 25% identity (51% similarity) with Yhr110w and 23% identity (46% similarity) with p24B. The structural relationship between these proteins, as summarized in Table 1, suggests that each represents a seperate member of a novel protein family. Common (structural) features found within this protein family include an N-terminal signal sequence, a conserved pair of cysteines (residues 13 and 74 in X1262), a transmembrane domain close to the C-terminus and a calculated molecular mass of ~22 kDa (excluding signal peptide). Single Asn-linked glycosylation sites are present in all proteins, except in p24B. Both the X1262 protein and its human homologue contain a strikingly high number of positively charged residues at their C-terminal ends. Included is a double lysine motif (residues 183 and 184 in X1262) that may function as a signal for the retrieval of transmembrane proteins to the ER [31,32]. The same motif is present in the gp25L protein, whereas two lysine residues at positions −3 and −5 from the Yhr110w C-terminus also match the consensus for this ER retrieval signal.

DISCUSSION

We have identified transcripts for TRAPδ and a novel protein similar to gp25L among a distinct set of messengers whose expression levels parallel that of POMC in physiologically activated *Xenopus* melanotropes. TRAPδ and gp25L are constituents of distinct tetrameric protein complexes that reside in the ER membrane within a short distance from translocating polypeptides.

TRAPδ was initially isolated by Migliaccio et al. [9] as part of a complex whose other components are TRAPα, -β and -γ.

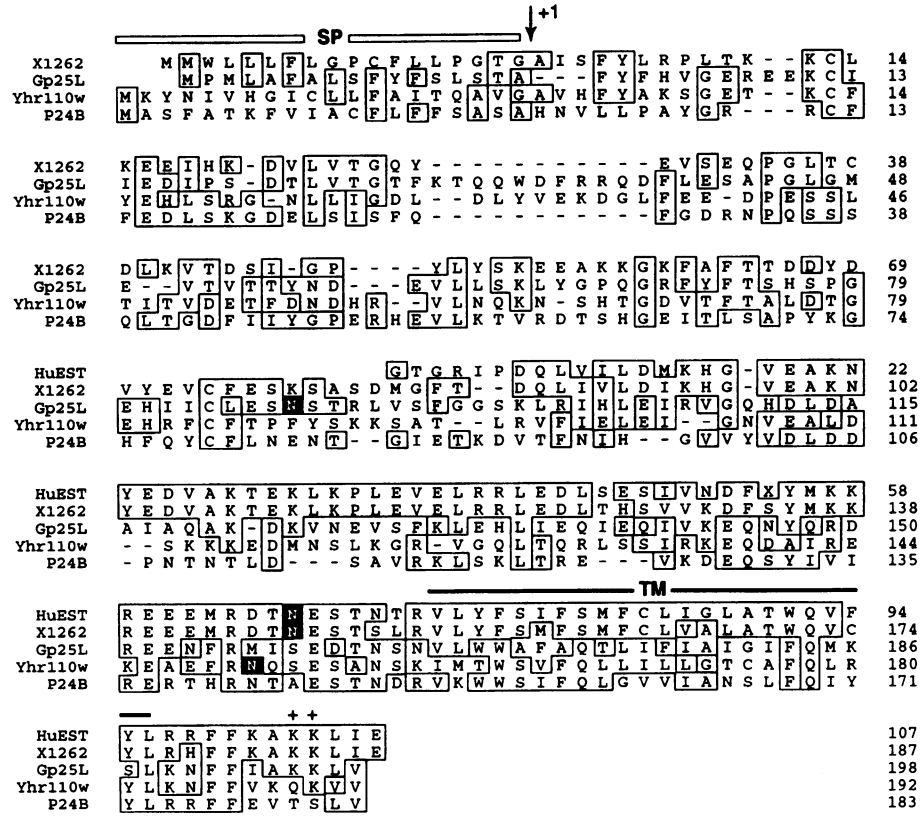

Figure 6 Alignment of amino acid sequences deduced from *Xenopus* cDNA X1262, human ESTs HuEST, dog glycoprotein gp25L and yeast proteins Yhr110w and p24B

The single-letter code is used. Gaps are introduced for optimal alignment. Potential Asn-linked glycosylation sites are indicated by white letters. Other designations are as in Figure 4. The HuEST sequence is a compilation of EMBL database entries HS23814 and HBC1303 (accession numbers T32283 and T10797). The dog gp25L protein sequence was taken from Wada et al. [11]. The yeast protein Yhr110w and p24B sequences were taken from the SwissProt database (accession numbers P38819 and P32803). The X1262 cDNA sequence is available from the EMBL database under accession number X90517.

Table 1 Sequence identity/similarity between gp25L-related proteins

	X1262	HuEST	gp25L	Yhr110w	P24B	
X1262	—	83	31	25	23	
HuEST	91	—	27	26	29	
gp25L	52	49	—	32	27	Identity (%)
Yhr110w	51	55	52	—	30	
P24B	46	51	42	49	—	
		Similarity (%)				

Cross-linking experiments have indicated that the four proteins are genuine neighbours in intact ER membranes [12]. TRAPδ is capable of forming homodimers and may connect two or more copies of the TRAP complex in the membrane. At present, the function of the TRAP complex is unknown. Sequence comparison between cDNAs from distantly related species (mammals, fish and nematodes) has revealed that TRAPα and TRAPβ are highly conserved proteins [12]. Our present findings demonstrate that the same applies for TRAPδ; the mature *Xenopus* protein and its mammalian counterparts share an overall sequence identity of ~ 80 %. Hence the TRAP complex probably serves a role that has been highly conserved during evolution. TRAPδ is encoded by a ubiquitously expressed gene, as in *Xenopus* the transcript is present in all 14 different tissues examined. In addition, we noticed that the TRAPδ transcript undergoes alternative splicing and as a result generates two structurally distinct proteins of ~14 kDa and ~17 kDa. Alternative splicing of the transcript was detected in a wide range of tissues and represents an evolutionarily conserved event that takes place in both amphibians and mammals. Migliaccio et al. [9] and Hartmann et al. [12] previously described only the 17 kDa form of TRAPδ; their studies did not contain any indication of the existence of a smaller form of this protein. A likely explanation for this would be that 17 kDa TRAPδ, but not the 14 kDa protein, is part of the TRAP complex. In that case, the region 22 amino acids long upstream of the transmembrane segment that is deleted in the 14 kDa protein could be essential for the interaction of TRAPδ with the other subunits of the complex. Apart from the high structural conservation of TRAPδ and alternative splicing of its transcript, the functional importance of this protein is underscored by a dynamic regulation of its gene in stimulated neuroendocrine cells (as discussed below).

In addition to TRAPδ mRNA, we identified a ubiquitously expressed *Xenopus* transcript for a novel and highly conserved protein with structural similarity to dog glycoprotein gp25L. Like gp25L, the *Xenopus* protein has a single transmembrane

segment positioned close to its C-terminus and a double lysine motif that may function as a retrieval signal for ER transmembrane proteins. Moreover, gp25L and the *Xenopus* gp25L-like protein are structurally related to yeast proteins Yhr110w and p24B. Yhr110w is a hypothetical type I membrane glycoprotein and also contains the ER retrieval signal. Its gene resides in the intergenic region CDC12–MSH1 of yeast chromosome VIII [33]. P24B is a type I membrane protein isolated from a crude preparation of yeast endosomes [34]. As the endosome-enriched fraction was found to contain several ER resident proteins, it remains to be established whether P24B is a true marker of the endosome. Altogether, our findings provide evidence that the gp25L-like protein identified in this study belongs to a novel family of ER-resident transmembrane proteins whose members are spread throughout the animal kingdom. At present, the functions of these proteins are unknown. Wada et al. [11] reported that gp25L is associated with calnexin and two subunits of the TRAP complex, namely TRAPα and TRAPβ. The authenticity of this protein complex was confirmed with different purification procedures and by co-immunoprecipitation. However, gp25L and calnexin were not found in the TRAP complex identified by Migliaccio et al. [9] and Hartmann et al. [12]. The isolation of the two different protein complexes may be due to differences in the purification protocols employed by these research groups. In particular, variations in the methods by which the membrane proteins were extracted from the microsomes could be important; such variations have previously caused conflicting results about the composition of the signal peptidase protein complex ([35] and references therein). Interestingly, Hartmann et al. [12] noted that the TRAP complex is stable in mild detergents such as cholate, and that upon application of stronger detergents (e.g. Nonidet P-40) only the interaction of TRAPα with TRAPβ remained intact. Hence it can be proposed that a tightly associated TRAPα–β dimer in proximity to the translocon may interact with different accessory subunits to form TRAP complexes of varying composition. Deshaies et al. [36] have presented results suggesting that also the integral components of the translocon in yeast exist as a dynamic assembly in the ER. Our identification of a gp25L-like protein suggests that TRAP complexes containing other components than those reported to date may indeed exist. The various assembly states of the TRAP proteins could meet differential requirements by subsets of precursor proteins or, alternatively, reflect cycles of subunit association and dissociation that mark discrete steps in the translocation event.

The data presented in this study suggest that the protein environment surrounding a translocating polypeptide in the ER is even more complex than previously expected, and possibly includes protein variants generated by alternative RNA splicing (e.g. 14 kDa and 17 kDa TRAPδ) as well as distinct members of protein families (e.g. gp25L and gp25L-like proteins). In addition, the genes for some of these proteins seem to be vigorously regulated in specialized secretory cells. In the *Xenopus* pituitary gland we observed marked cell-type-specific changes in the transcript levels for TRAPδ and the gp25L-like protein during the physiological process of background adaptation. Similar dynamics in gene expression have been observed for the prohormone POMC [14], prohormone-processing enzymes (PC2 and CPH) [37] and several additional constituents of secretory granules (SGII, SGIII and 7B2) ([38] and J. Holthuis, E. Jansen, M. van Riel and G. Martens, unpublished work). The biosynthetic machinery in activated *Xenopus* melanotropes is primarily dedicated to the production of POMC-derived peptide hormones: POMC or POMC-derived cleavage products constitute up to 80% of all newly synthesized proteins in these cells (J. Holthuis, E. Jansen, M. van Riel and G. Martens, unpublished work). Taken together, these data suggest that in *Xenopus* melanotropes the TRAP complex, or subunits thereof, are important for the biosynthesis of POMC either during or shortly after the emergence of the prohormone in the ER lumen. One could think of a general role in the unidirectional transfer of nascent polypeptides across the ER membrane, in the proper release of newly synthesized polypeptides from the translocation site or in a hithertho unrecognized protein modification step. It is also conceivable that these proteins participate in the folding or quality control systems of the ER. It has been well established that on hyperstimulation of exocrine and endocrine cells, regulated secretory proteins entering the ER tend to condensate into intracisternal granules from which other soluble ER proteins are excluded [39,40]. This premature condensation, which has also been observed in stimulated *Xenopus* melanotropes [41], is thought to develop when export of regulated secretory proteins from the ER fails to keep pace with the rate of their synthesis. Under these circumstances, the folding and quality control systems of the ER are likely to enter a highly alarmed state in which the production rates of the components involved will be elevated.

Note added in proof (received 9 October 1995)

A recent report has shown that the yeast P24B protein is a component of ER-derived COPII-coated vesicles and has a role in the correct delivery of a subset of secretory proteins to the Golgi complex [42].

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REFERENCES

1 Deshaies, R. J., Koch, B. D., Werner-Washburne, M., Craig, E. A. and Schekman, R. (1988) Nature (London) **332**, 800–805
2 Rothblatt, J. A., Deshaies, R. J., Sanders, S. L., Daum, G. and Schekman, R. (1989) J. Cell Biol. **109**, 2641–2652
3 Görlich, D. and Rapoport, T. A. (1993) Cell **75**, 615–630
4 Görlich, D., Hartmann, E., Prehn, S. and Rapoport, T. A. (1992) Nature (London) **357**, 47–52
5 Wiedman, M., Kurzchalia, T. V., Hartmann E. and Rapoport, T. A. (1987) Nature (London) **328**, 830–833
6 Prehn, S., Herz, J., Hartmann, E., Kurzchalia, T. V., Frank, R., Römisch, K., Dobberstein, B. and Rapoport, T. A. (1990) Eur. J. Biochem. **188**, 439–445
7 Collins, P. G. and Gilmore, R. (1991) J. Cell Biol. **114**, 639–649
8 Hartmann, E., Wiedmann, M. and Rapoport, T. A. (1989) EMBO J. **8**, 2225–2229
9 Migliaccio, G., Nicchitta, C. V. and Blobel, G. (1992) J. Cell Biol. **117**, 15–25
10 Görlich, D., Prehn, S., Hartmann, E., Herz, J., Otto, A., Kraft, R., Wiedmann, M., Knespel, S., Dobberstein, B. and Rapoport, T. A. (1990) J. Cell Biol. **111**, 2283–2294
11 Wada, I., Rindress, D., Cameron, P. H. et al. (1991) J. Biol. Chem. **266**, 19599–19610
12 Hartmann, E., Görlich, D., Kosta, S., Otto, A., Kraft, R., Knespel, S., Bürger, E., Rapoport, T. A. and Prehn, S. (1993) Eur. J. Biochem. **214**, 375–381
13 Jenks, B. G., Overbeeke, A. P. and McStay, B. F. (1977) Can. J. Zool. **55**, 922–927
14 Martens, G. J. M., Weterings, K. A. P., van Zoest, I. D. and Jenks, B. G. (1987) Biochem. Biophys. Res. Commun. **143**, 678–684
15 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
16 Rodriguez, I. R. and Chader, G. J. (1992) Nucleic Acids Res. **20**, 3528
17 van Riel, M. C. H. M., Tuinhof, R., Roubos, E. W. and Martens, G. J. M. (1993) Biochim. Biophys. Acta **190**, 948–951

18 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
19 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
20 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1989) Current Protocols in Molecular Biology, John Wiley, New York
21 Holthuis, J. C. M., Schoonderwoert, V. T. G. and Martens, G. J. M. (1994) Biochim. Biophys. Acta **1219**, 184–188
22 Kozak, M. (1989) J. Cell Biol. **108**, 229–241
23 Heijne, G. von (1986) Nucleic Acids Res. **14**, 4683–4690
24 Whali, W., Dawid, I. B., Wyler, T., Jaggi, R. B., Weber, R. and Ryffel, G. U. (1979) Cell **16**, 535–549
25 Westley, B. R., Wyler, T., Ryffel, G. U. and Weber, R. (1981) Nucleic Acids Res. **9**, 3557–3574
26 Martens, G. J. M. and Herbert, E. (1984) Nature (London) **310**, 251–254
27 Loreni, F., Ruberti, I., Bozzoni, I., Pierandrei-Amaldi, P. and Amaldi, F. (1985) EMBO J. **4**, 3483–3488
28 Martens, G. J. M. (1986) Nucleic Acids Res. **14**, 3791–3798
29 Bisbee, C. A., Baker, M. A., Wilson, C. A. Hadji-Azimi, I. and Fischberg, M. (1977) Science **195**, 785–787
30 Thiebaud, C. H. and Fishberg, M. (1977) Chromosoma **59**, 253–257
31 Jackson, M. R., Nilsson, T. and Peterson, P. A. (1990) EMBO J. **9**, 3153–3162
32 Jackson, M. R., Nilsson, T. and Peterson, P. A. (1993) J. Cell Biol. **121**, 317–333
33 Johnston, M., Andrews, S., Brinkman, R. et al. (1994) Science **265**, 2077–2082
34 Singer-Krüger, B., Frank, R., Crausaz, F. and Riezman, H. (1993) J. Biol. Chem. **268**, 14376–14386
35 Shelness, G. S., Kanwar, Y. S. and Blobel, G. (1988) J. Biol. Chem. **263**, 17063–17070
36 Deshaies, R. J., Sanders, S. L., Feldheim, D. A. and Schekman, R. (1991) Nature (London) **349**, 806–808
37 Braks, J. A. M., Guldemond, K. C. W., van Riel, M. C. H. M., Coenen, A. J. M. and Martens, G. J. M. (1992) FEBS Lett. **305**, 45–50
38 Martens, G. J. M., Bussemakers, M. J. G., Ayoubi, T. A. Y. and Jenks, B. G. (1989) Eur. J. Biochem. **181**, 75–79
39 Farquhar, M. G. (1971) In Sub-cellular Structure and Function in Endocrine Organs (Heller, H. and Lederis, K., eds.), pp. 79–122, Cambridge University Press, Cambridge
40 Tooze, J., Kern, H. F., Fuller, S. D. and Howell, K. E. (1989) J. Cell Biol. **109**, 35–50
41 Hopkins, C. R. (1972) J. Cell Biol. **53**, 642–653
42 Schimmöller, F., Singer-Krüger, B. Schröder, S., Krüger, U., Barlowe, C. and Riezman, H. (1995) EMBO J. **14**, 1329–1339

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