

μ 1B, a novel adaptor medium chain expressed in polarized epithelial cells¹

Hiroshi Ohno^{a,*}, Takuya Tomemori^c, Fubito Nakatsu^a, Yasushi Okazaki^a,
Ruben C. Aguilar^b, Heike Foelsch^d, Ira Mellman^d, Takashi Saito^a, Takuji Shirasawa^c,
Juan S. Bonifacino^{b,†}

^a Department of Molecular Genetics, Chiba University Graduate School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan

^b Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Building 18T, Room 101, 18 Library Dr. MSC 5430, Bethesda, MD 20892-5430, USA

^c Department of Molecular Genetics, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173-0015, Japan

^d Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06520-8002, USA

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Abstract The apical and basolateral plasma membrane domains of polarized epithelial cells contain distinct sets of integral membrane proteins. Biosynthetic targeting of proteins to the basolateral plasma membrane is mediated by cytosolic tail determinants, many of which resemble signals involved in the rapid endocytosis or lysosomal targeting. Since these signals are recognized by adaptor proteins, we hypothesized that there could be epithelial-specific adaptors involved in polarized sorting. Here, we report the identification of a novel member of the adaptor medium chain family, named μ 1B, which is closely related to the previously described μ 1A (79% amino acid sequence identity). Northern blotting and *in situ* hybridization analyses reveal the specific expression of μ 1B mRNA in a subset of polarized epithelial and exocrine cells. Yeast two-hybrid analyses show that μ 1B is capable of interacting with generic tyrosine-based sorting signals. These observations suggest that μ 1B may be involved in protein sorting events specific to polarized cells.

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1. Introduction

The plasma membrane of polarized epithelial cells is differentiated into apical and basolateral domains, each of which contains a distinct complement of membrane proteins (reviewed in [1,2,3]). The segregation of membrane proteins into these domains occurs by sorting events that take place at the *trans*-Golgi network (TGN) and/or endosomes [1,2,3]. Sorting to the apical domain is thought to involve incorporation of proteins into sphingolipid- and cholesterol-rich membrane 'rafts' [4] or interaction with lectin-like sorting receptors [5,6]. In contrast, sorting to the basolateral domain is largely determined by specific sorting signals contained within the cytosolic domains of the proteins [1,2]. Basolateral sorting

signals are highly degenerate, although many of them resemble tyrosine-based or di-leucine-based motifs involved in rapid endocytosis and lysosomal targeting [1,2]. Other basolateral sorting signals are co-linear with but distinct from these motifs [7,8] and some do not conform to any recognizable canonical sequence [9–12].

The resemblance of many basolateral sorting signals to endocytic and lysosomal targeting signals suggests that they may be recognized by similar components of the sorting machinery. It is now well-established that both tyrosine-based and di-leucine-based signals involved in endocytosis and lysosomal targeting are recognized by the heterotetrameric adaptor complexes AP-1 (γ - β 1- μ 1- σ 1), AP-2 (α - β 2- μ 2- σ 2) and AP-3 (δ - β 3- μ 3- σ 3) (reviewed in [13–17]). The medium subunits μ 1, μ 2 and μ 3 of the respective adaptor complexes are directly responsible for the recognition of tyrosine-based sorting signals [18–22]. Di-leucine-based signals have been shown to interact with μ 1 and μ 2 [23,24] as well as with β 1 [25]. These observations led us to hypothesize that sorting signals that function specifically in polarized epithelial cells might likewise be recognized by adaptor proteins.

Here, we report the identification of a novel member of the adaptor medium chain family, termed μ 1B, which is specifically expressed in polarized epithelial cells and some exocrine cells. μ 1B is most closely related to the ubiquitously-expressed μ 1A subunit of AP-1 (79% identity at the amino acid level). Two-hybrid assays demonstrate that μ 1B is capable of interacting with generic tyrosine-based sorting signals, consistent with the possibility that μ 1B may be an adaptor protein involved in protein sorting in polarized cells.

2. Materials and methods

2.1. Cloning of human and mouse μ 1B cDNAs

A human EST clone from the I.M.A.G.E. Consortium (LLNL, ID 123283, [26]) encoding a portion of a protein closely related to the known mouse μ 1 (GenBank #M62419, [27]) was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). A human placenta cDNA library (Clontech Laboratories, Palo Alto, CA, USA) was screened using the insert from the EST clone as a probe in order to obtain a full length cDNA. A mouse clone (ID 372139) highly homologous to human μ 1B was also obtained from the ATCC. The missing 5'-part of the encoding region was obtained from mouse kidney RNA by a 5'-RACE PCR, using the SMART PCR cDNA Synthesis kit (Clontech Laboratories).

2.2. *In vitro* transcription/translation

³⁵S-labelled mouse μ 1A, human μ 1B and mouse μ 2 were prepared

*Corresponding author. Fax: (81) (43) 222 1791.

E-mail: ohno@gene.m.chiba-u.ac.jp

†Second corresponding author. Juan S. Bonifacino, Fax: (1) (301) 402 0078. E-mail: juan@helix.nih.gov

¹ The nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers AF020797 (human μ 1B) and AF067146 (mouse μ 1B).

Abbreviations: EST, expressed sequence tag; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; TGN, *trans*-Golgi network

A

Hmu 1B	1	MSASAVFILDVKGKPLISRNYKGDVA	MSKIEHFMPPLLVQREEEGAL	LAPLLSHGQ	VHFLWI
Mmu 1B	1	MSASAVFILDVKGKPLISRNYKGDV	MTTEIDHFMPPLLMQREEEGVL	LAPLLSHGR	VHFLWI
Mmu 1A	1	MSASAVYVLDLKGKVLICRNYR	MSVEVEHFMPPLLMKREEEGML	SPILLAHG	GVRFFMWI
Hmu 1B	61	KHSNLYLVATTS	KNANASLVYSFLYKTI	EVFCEYFKELEEE	SIRDNV
Mmu 1B	61	KHSNLYLVATTL	KNANASLVYSFLYKTV	EVFCEYFKELEEE	SIRDNF
Mmu 1A	61	KHNLYLVATSK	KNACVSLVFSFLYKVV	QVFSSEYFKELEEE	SIRDNF
Hmu 1B	121	GFPQTTDSKILQEYITQQ	SNKLETGKSRVPPTVT	NAVSWRSEGIKYK	KNEVFIDVIESVN
Mmu 1B	121	GFPQTTDSKILQEYITQQ	GKNKLETGKSRVPPTVT	NAVSWRSEGIKYK	KNEVFIDVIESVN
Mmu 1A	121	GVPQTTDSKILQEYITQE	GKLETGAPRPPATVT	NAVSWRSEGIKYR	KNEVFIDVIEAVN
Hmu 1B	181	LLVNANGSVLLSEIVGTIKLVFLSGMPELRLGLNDRVLF	ELTGRSKNKSVE	LEDVKFHQ	
Mmu 1B	181	LLVNANGSVLLSEIVGTIKLVFLSGMPELRLGLNDRVLF	ELTGRSKNKSVE	LEDVKFHQ	
Mmu 1A	181	LLVSANGNVLRLSEIVGSIKMRVFLSGMPELRLGLNDKVLFD	NTTGRGKS	KSVELEDVKFHQ	
Hmu 1B	241	CVRLSRFDNDRTISFIPPDGDFELMSYRLSTQVKPLIWIESVIEKFS	HSRVEIMVKAKGQ		
Mmu 1B	241	CVRLSRFDNDRTISFIPPDGDFELMSYRLSTQVKPLIWIESVIEKFS	HSRVEIMVKAKGQ		
Mmu 1A	241	CVRLSRFNDNDRTISFIPPDGDFELMSYRLNTHTVVKPLIWIESVIEKFS	HSRTEVMVKAKSQ		
Hmu 1B	301	FKKQSVANGVEISVPVPSDADS	PRFKTSVGS	AKYVPERNV	VIWSIKSFP
Mmu 1B	301	FKKQSVANGVEISVPVPSDADS	PRFKTSVGS	AKYVPERNV	VIWSIKSFP
Mmu 1A	301	FKRRSTANVVEIHTTPV	PNDADSPKFKT	TVGSAK	YVPEKNNVIWSIKSFP
Hmu 1B	361	GLPSVEKEEVEGRPPIGVKFEI	PFYFTVSGIQVRYMKIIEK	SGYQALPWVRYITQ	SGDYQL
Mmu 1B	361	GLPSVETEVEVEGRPPIGVKFEI	PFYFTVSGIQVRYMKIIEK	SGYQALPWVRYITQ	SGDYQL
Mmu 1A	361	GLPSVEAEDEKEGKPPISV	KFEIPFYFTTSGIQVRY	KIIEKSGYQALPWVRYITQ	NGDYQL
Hmu 1B	421	RTS			
Mmu 1B	421	RTS			
Mmu 1A	421	RTQ			

B

	μ 1B (human)		μ 1B (mouse)	
	% I	% S	% I	% S
μ 1B (human)	100	100	97	98
μ 1B (mouse)	97	98	100	100
μ 1A (mouse)	79	90	80	91
μ 2 (mouse)	38	65	38	65
μ 3A (rat)	30	57	29	57
μ 3B (rat)	28	55	28	55
μ 4 (human)	29	53	28	53

C

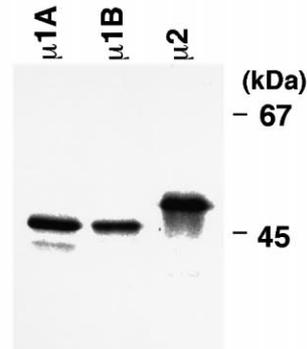


Fig. 1. Sequence analysis and characterization of μ 1B. (A) Alignment of human μ 1B (AF020797), mouse μ 1B (AF067146) and mouse μ 1A (W08014) amino acid sequences. Residues that are identical in at least two sequences are boxed. (B) Sequence identity (I) and similarity (S) of different members of the adaptor μ chain family. (C) SDS-PAGE analysis of in vitro transcribed/translated, [35 S]methionine-labelled mouse μ 1A, human μ 1B and mouse μ 2. The positions of molecular size markers are indicated on the right.

using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA) in the presence of [35 S]methionine (Tran 35 S-Label, ICN, Costa Mesa, CA, USA). An aliquot of each product was directly mixed with Laemmli sample buffer, separated by SDS-PAGE and analyzed with a Fujix Bio-imaging Analysis System.

2.3. Cell lines

Rat basophilic leukemia cells (clone 2H3) were kindly provided by Dr Henry Metzger (National Institutes of Health, Bethesda, MD, USA). MDCK II (canine kidney epithelial) and Caco-2 (human colon adenocarcinoma) cells were kindly provided by Drs Sachiko and Shochiro Tsukita (Kyoto University, Kyoto, Japan). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, 100 μ g/ml streptomycin (Sigma Chemical, St. Louis, MO, USA). The following cells

were obtained from the ATCC and cultured as recommended by the supplier: HT-29 (human colon adenocarcinoma), HEC-1-A (human endometrial adenocarcinoma), RL95-2 (human endometrial adenocarcinoma), AV-3 (human amnion epithelial), VERO C1008 (African green monkey kidney), LLC-PKI (porcine kidney epithelial), HeLa (human cervical carcinoma), Jurkat (human acute T-cell leukemia), JY (human B lymphoblastoma), A-549 (human lung carcinoma) and HuTu 80 (human duodenum adenocarcinoma).

2.4. Northern analyses

Total RNA was extracted from cultured cells and various mouse tissues according to Chomczynski and Sacchi [28]. 10 μ g of RNA was electrophoresed on 1% agarose gels containing 0.22 M formaldehyde and transferred to Nylon membranes (Hybond N⁺, Amersham International, Buckinghamshire, UK). Human Multiple Tissue Northern

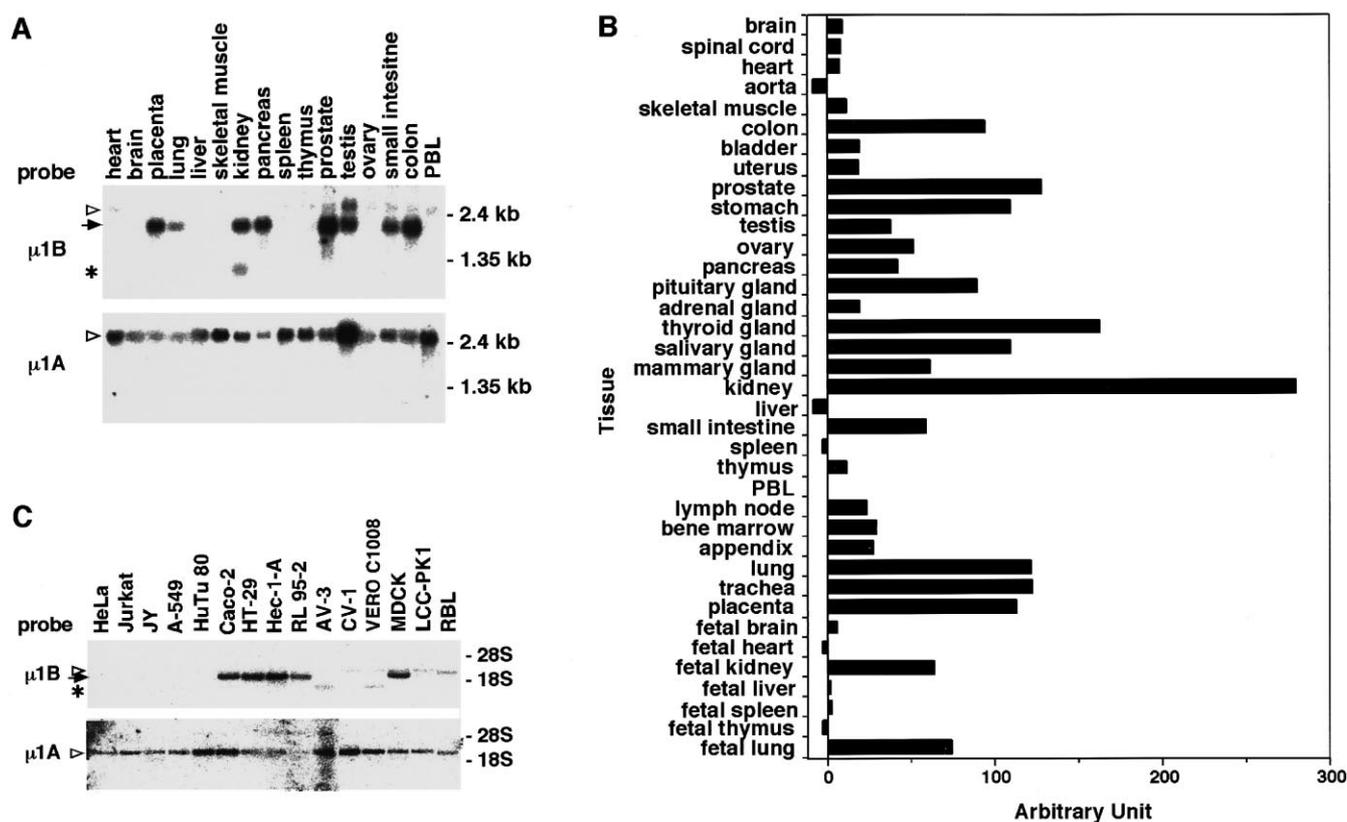


Fig. 2. Analyses of $\mu 1B$ mRNA expression. (A) Human multiple tissue Northern blots probed with ^{32}P -labelled human $\mu 1B$ and $\mu 1A$ cDNAs. Positions of $\mu 1B$ (arrow) and $\mu 1A$ (open triangles) transcripts are indicated on the left. The asterisk indicates a faster migrating $\mu 1B$ RNA species detected in kidney. The positions of RNA size markers are indicated on the right. (B) Quantitation of a human RNA dot blot probed with ^{32}P -labelled human $\mu 1B$ cDNA. (C) Total RNAs (10 μ g) from various cultured cell lines probed with ^{32}P -labelled human $\mu 1B$ and $\mu 1A$ cDNAs. The positions of $\mu 1B$ (arrow) and $\mu 1A$ (open triangles) transcripts are indicated on the left. The asterisk indicates a faster migrating $\mu 1B$ RNA species detected in the lanes corresponding to AV-3 and VERO C1008 cells. The positions of the 18S and 28S rRNAs are indicated on the right.

(MTN) Blots I and II, as well as Human RNA Master Blot, were purchased from Clontech. For human RNA samples, Nylon membranes were first probed with ^{32}P -labelled human $\mu 1B$ cDNA (a PCR fragment corresponding to nucleotides 757–1363 of human $\mu 1B$, GenBank AF020797). After stripping off the probe, membranes were re-probed with ^{32}P -labelled human $\mu 1A$ cDNA (the insert of an EST clone, ID hbc4916, containing a segment of the human $\mu 1A$ cDNA). Mouse RNA samples were sequentially probed with mouse $\mu 1B$ (a *Pst* I fragment corresponding to nucleotides 391–1313, GenBank AF067146) and $\mu 1A$ (a PCR fragment corresponding to nucleotides 73–342, GenBank W08014) cDNA fragments.

2.5. In situ hybridization

C57BL/6 mouse embryos at day 16 p.c. were fixed in 4% paraformaldehyde in PBS at 4°C overnight, dehydrated with ethanol and embedded in paraffin. Serial sections of 5 μ m were cut and mounted on poly-L-lysine coated slides. After removal of paraffin, sections were post-fixed in 4% paraformaldehyde, treated with 0.25% acetic anhydride in 0.1 M triethanolamine and dehydrated again. Procedures for in situ hybridization were essentially as described [29]. For the antisense probe of mouse $\mu 1B$, pGEM3Zf(+) plasmid (Promega, Madison, WI, USA) harboring a 922 bp *Pst* I fragment (nucleotides 369–1291) from mouse $\mu 1B$ cDNA was linearized with *Hind* III and transcribed with T7 polymerase according to the manufacturer's instructions (Promega, Madison, WI, USA) with [^{35}S]UTP (740 MBq/ml, Amersham). The sense probe was linearized with *Bam* HI and transcribed with SP6 polymerase. Hybridization, washing and exposure were performed as previously described [29]. Sections were counterstained with hematoxylin and eosin (H and E). Dark-field images were superimposed with bright-field images, modified with color filters and photographed.

2.6. Yeast two-hybrid analysis

YXX \emptyset signals fused to GAL4bd were derived from a random combinatorial library prepared for analyzing specificity μ chain interactions with tyrosine-based signals [20]. GAL4ad- $\mu 1B$ was made by ligation of a *Sal* I-*Xho* I fragment from a $\mu 1B$ cDNA clone into the *Xho* I site of the vector pACT2. Other GAL4ad- μ chain constructs and procedures of the two-hybrid analyses were described previously [18–20].

3. Results and discussion

A human cDNA encoding a novel μ chain was cloned based on sequence information found in EST databases. The cDNA encoded a protein of 423 amino acids and a predicted molecular mass of 48 030 Da. The novel protein was most closely related to mouse $\mu 1$ (79% identity at the amino acid sequence level) (Fig. 1A) and more distantly related to other members of the adaptor medium chain family (30–40% identity to $\mu 2$, $\mu 3A$, $\mu 3B$ and μ -ARP2 ($\mu 4$, [30]) and ~20% identity to δ -COP [31]) (Fig. 1B). We also cloned a mouse cDNA encoding the murine homolog of the human protein (97% identity at the amino acid level) (Fig. 1A), thus demonstrating that the novel protein corresponds to a conserved gene product distinct from the previously described $\mu 1$. Because $\mu 1$ and the novel protein were distinct but closely related, they were designated $\mu 1A$ and $\mu 1B$, respectively. The $\mu 1A$ and $\mu 1B$ polypeptides produced by in vitro transcription/

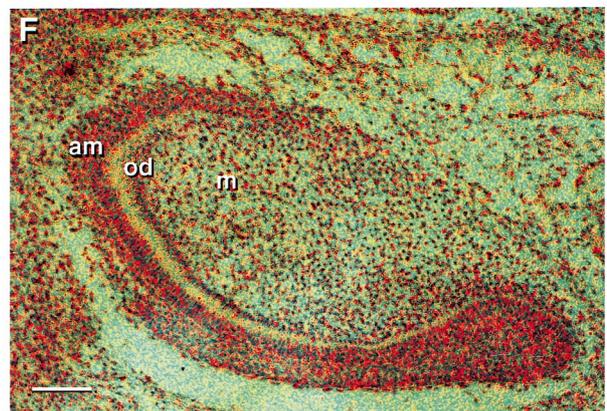
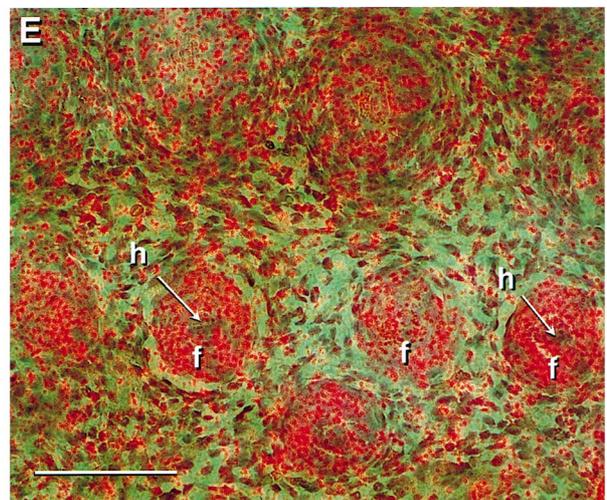
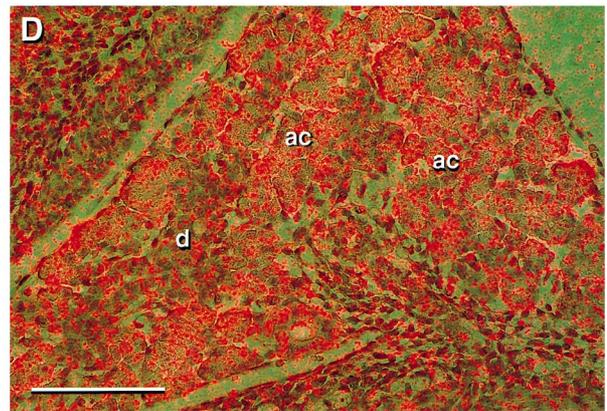
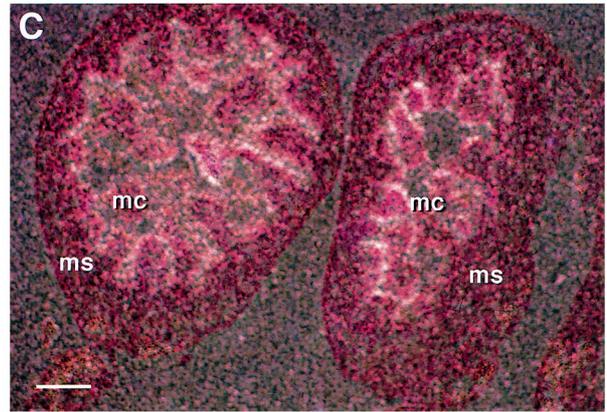
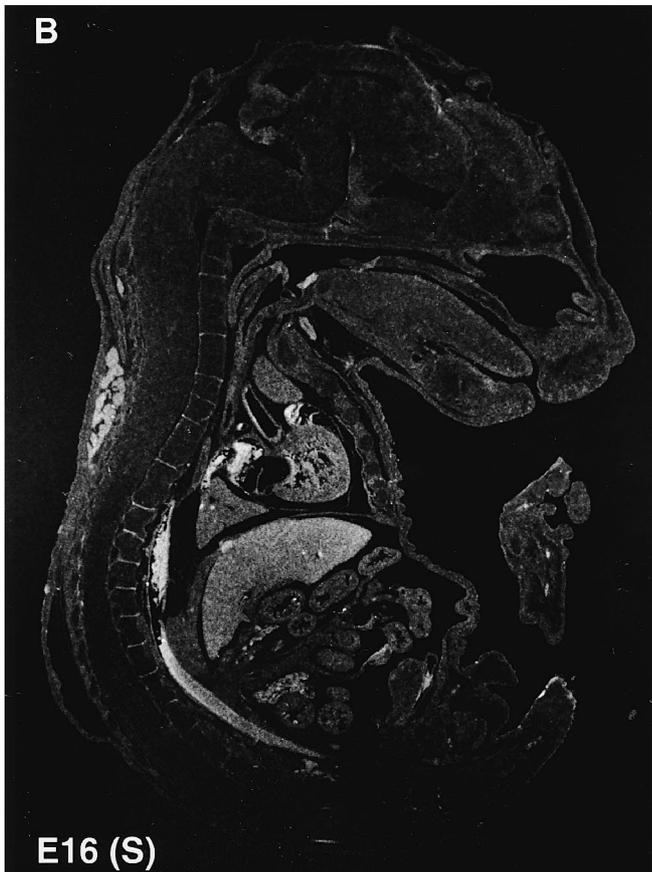
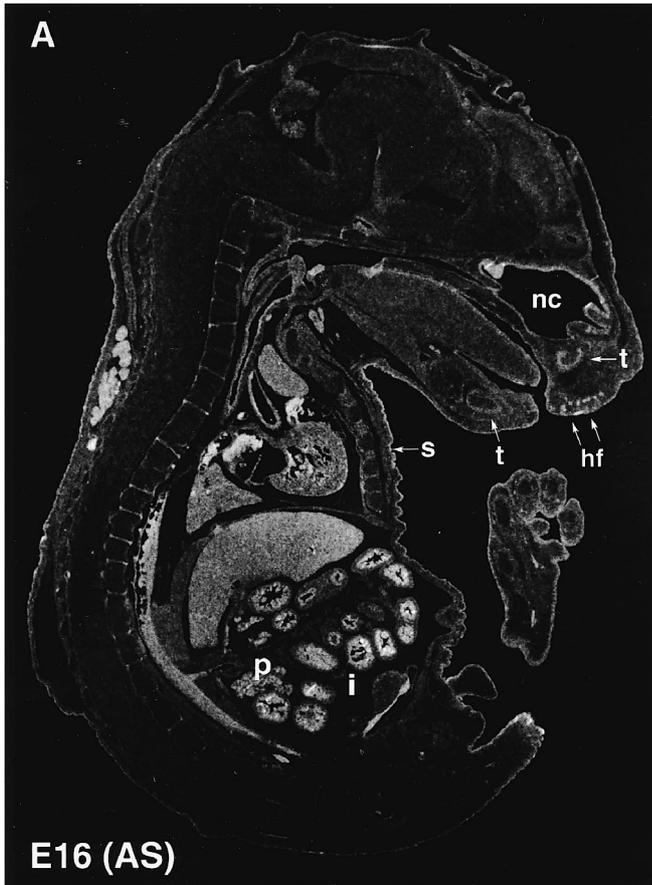


Fig. 3. In situ hybridization of $\mu 1B$ mRNA in mouse embryonal tissues. (A, B) Parasagittal sections of E16 mouse embryo, hybridized with antisense (A) or sense (B) RNA probes for $\mu 1B$. Intense signals are detected in the intestine (i) whereas moderate signals are found in the pancreas (p), hair follicles (hf), tooth primordia (t) and nasal cavity (nc). (C–F) Sections of fetal intestine (C), fetal pancreas (D), fetal hair follicles (E) and a fetal tooth bud (F), hybridized with antisense RNA probes for $\mu 1B$ and counterstained with H and E. (C) Scale bar, 200 μm . (D–F) Scale bar, 100 μm .

translation co-migrated on SDS-PAGE with an apparent molecular mass of ~ 47 kDa (Fig. 1C).

Northern analyses revealed that $\mu 1B$ mRNA was expressed in only some human tissues, including placenta, lung, kidney, pancreas, prostate, testis, small intestine and colon (Fig. 2A), in contrast to $\mu 1A$ mRNA which was expressed in all human tissues examined (Fig. 2A). In addition, dot blot analyses demonstrated a high level expression of $\mu 1B$ mRNA in stomach, pituitary gland, salivary glands and trachea (Fig. 2B). The same pattern of differential expression of $\mu 1A$ and $\mu 1B$ mRNAs was observed in mouse tissues (data not shown). A common feature of all the tissues that express $\mu 1B$ mRNA is the presence of epithelial and/or exocrine cell-types, both of which are polarized. To investigate whether $\mu 1B$ mRNA was specifically expressed in polarized cell-types, we performed Northern analyses of various cultured cell lines (Fig. 2C). These experiments demonstrated expression of $\mu 1B$ mRNA in Caco-2, HT-29, Hec-1-A, RL 95-2 and MDCK cells, all epithelial cells that become polarized in culture. Some epithelial cells (e.g. LLC-PK1) as well as non-epithelial cells expressed little or no $\mu 1B$ mRNA, although they all expressed $\mu 1A$ mRNA (Fig. 2C).

To analyze the spatial expression of the $\mu 1B$ gene in mouse tissues, we performed in situ hybridization on mouse embryos. In a parasagittal section of day 16 p.c. embryos, the highest levels of $\mu 1B$ mRNA were observed in the gastrointestinal mucosa (Fig. 3, compare A and B). Moderate levels of $\mu 1B$ gene expression were detected in the pancreas, hair follicles, tooth buds and nasal cavity (Fig. 3A and B). In the gastrointestinal tract, $\mu 1B$ mRNA was highly expressed in the intestinal mucosa (mc) but was barely detected in the submucosa and muscle layer (ms) (Fig. 3C). In the pancreas, strong in situ signals were observed in acinar cells (ac) but not in the intercalated duct cells (d) (Fig. 3D). $\mu 1B$ mRNA was also specifically expressed in epithelial cells of hair follicles (Fig. 3E), as well as in ameloblasts (am) and odontoblasts (od), polarized epithelial cells that secrete enamel and dentin, respectively, in developing teeth (Fig. 3F). These results confirmed that $\mu 1B$ mRNA is predominantly expressed in polarized epithelial cells and exocrine cells.

Next, we tested whether $\mu 1B$ was capable of interacting with cytosolic sorting signals using the yeast two-hybrid system [18–20]. Yeast cells were co-transformed with GAL4ad- $\mu 1B$ and GAL4bd fused to generic YXX \emptyset -type tyrosine-based sorting signals [20]. Like other members of the μ chain family, $\mu 1B$ was found to interact with a subset of YXX \emptyset -type signals in a tyrosine-dependent manner, as demonstrated by the growth of co-transformed yeast cells on medium lacking histidine (Fig. 4). This interaction is consistent with the fact that some YXX \emptyset -type signals function as basolateral sorting signals [32–36] and suggests that $\mu 1B$ could play a role in the recognition of YXX \emptyset signals in polarized cells.

The recent resolution of the crystal structure of $\mu 2$ [37] makes it possible now to analyze the structural bases for recognition of tyrosine-based sorting signals by members of the

adaptor medium chain family. Interestingly, of 11 residues in $\mu 2$ shown to be involved in the recognition of YXX \emptyset signals [37], seven are conserved and four are not conserved in $\mu 1B$. Among the conserved residues are D¹⁷⁶, F¹⁷⁴, L²⁰³, W⁴²¹, R⁴²³ of $\mu 2$, all of which are involved in interactions with the critical Y residue of YXX \emptyset signals [37]. In contrast, only two (i.e. V⁴⁰¹ and V⁴²²) of the six $\mu 2$ residues involved in interactions with the \emptyset side chain of YXX \emptyset signals (i.e. L¹⁷³, L¹⁷⁵, V⁴⁰¹, L⁴⁰⁴, K⁴²⁰, V⁴²², [37]) are conserved in $\mu 1B$. The substitution of I⁴¹⁹ in $\mu 2$ by L in $\mu 1B$ could also affect the recognition of residues at the second X position (Y+2). These structural considerations predict that $\mu 1B$ should be able to interact with a subset of tyrosine-based sorting signals, albeit with an affinity and/or fine specificity distinct from that of $\mu 2$. This prediction is fulfilled by the ability of $\mu 1B$ to bind only some generic sorting signals (Fig. 4), all of which bind to $\mu 2$ [20].

The specific expression of $\mu 1B$ mRNA in polarized epithelial and exocrine cells suggests that this protein might be involved in sorting events necessary for the establishment of cell polarity or for delivery of proteins to different plasma membrane domains. In this regard, it is noteworthy that the porcine kidney epithelial cell line LLC-PK1, which becomes polarized in culture, does not express detectable levels of $\mu 1B$ mRNA (Fig. 2C). Remarkably, LLC-PK1 cells have been shown to misroute two proteins having tyrosine-based signals, the H,K ATPase β subunit and an influenza hemagglutinin construct bearing a tyrosine-based signal, to the apical surface [38]. This is in contrast to MDCK cells, which express $\mu 1B$ mRNA (Fig. 2C) and sort both the H,K ATPase β subunit and the influenza hemagglutinin construct to the basolateral surface [38]. Since LLC-PK1 cells are otherwise polarized, these observations lend us support to the idea that $\mu 1B$ may be required for basolateral targeting.

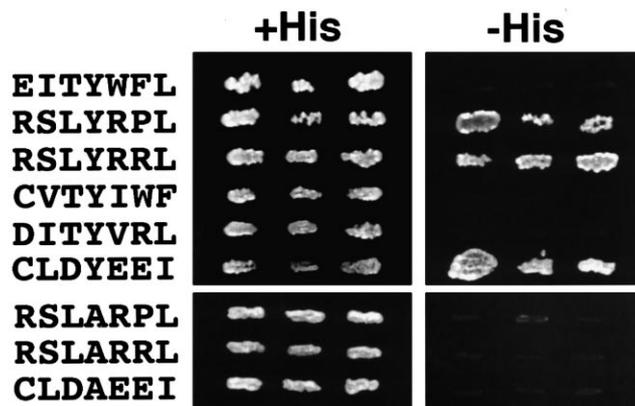


Fig. 4. Interactions of $\mu 1B$ with sorting signals. Interactions with YXX \emptyset signals. HF7c yeast cells were co-transformed with a plasmid encoding GAL4ad fused to human $\mu 1B$ and plasmids encoding GAL4bd fused to sequences of various YXX \emptyset signals and their Y to A mutants. Co-transformants were tested for their ability to grow in the presence (+His) or absence (-His) of histidine, as previously described [18].

The similarity of $\mu 1B$ to $\mu 1A$ makes it likely that $\mu 1B$ is an alternative component of the AP-1 clathrin-associated adaptor complex in polarized epithelial cells. We have been unable to test if $\mu 1B$ is indeed a component of AP-1, as the antibodies that were raised against $\mu 1B$ did not discriminate $\mu 1B$ from $\mu 1A$ (data not shown). Nonetheless, a role for AP-1 and clathrin in the basolateral targeting has been recently proposed by Futter et al. [39], who showed that vesicles bound at the basolateral domain bud from clathrin- and AP-1-coated endosomal tubules. A similar process may take place at the TGN, where AP-1 and clathrin are also located [40]. In this regard, Orzech et al. [41] have recently presented evidence that AP-1 interacts with a basolateral targeting determinant in the cytosolic tail of the polymeric immunoglobulin receptor, an interaction which may mediate sorting of the receptor at the TGN. An intriguing implication of our findings is that other polarized cells such as hepatocytes or neurons, which do not seem to express $\mu 1B$, may utilize other adaptor proteins for sorting to the basolateral equivalent of the plasma membrane. This is consistent with the idea that the mechanisms of polarized sorting in hepatocytes, neurons and epithelial cells are related but not identical [2,42].

In conclusion, our results suggest that $\mu 1B$ may be a component of the protein sorting machinery in polarized epithelial and exocrine cells. The information presented here should now enable further analyses of the role of $\mu 1B$ in protein sorting *in vivo*.

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