Identification and characterization of a novel splicing variant of vesicular monoamine transporter 1

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Abstract

Vesicular monoamine transporter 1 (VMAT1) is an integral protein in the membrane of secretory vesicles of neuroendocrine and endocrine cells that allows the transport of biogenic monoamines, such as serotonin, from the cytoplasm into the secretory vesicles. The full-length VMAT1 transcript is produced from 16 exons. We have identified and characterized an alternatively spliced form of VMAT1 that lacks exon 15, the next to last exon of VMAT1. The new form was therefore denoted VMAT1Δ15. Exon 15 does not contain an even multiple of three nucleotides. As a consequence, there is a shift of reading frame, and exon 16 is translated in an alternative reading frame, yielding a novel protein with a shorter and unrelated C-terminus compared with the native VMAT1 protein. VMAT1 and VMAT1Δ15 mRNAs are simultaneously expressed in normal and neoplastic neuroendocrine cells of the GI tract. However, VMAT1 expression is always higher than VMAT1Δ15 expression. We prove that VMAT1Δ15 is not localized in large, dense core vesicles as the native form but in the endoplasmic reticulum. Furthermore, while VMAT1 can take up serotonin, VMAT1Δ15 cannot, indicating different functions for the two forms of VMAT1.

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Introduction

Enterochromaffin cells (EC) are specialized neuroendocrine cells, scattered throughout the gastrointestinal epithelium. They synthesize, store and release the biogenic amine serotonin (5-hydroxytryptamine (5-HT)) as well as a variety of other peptide hormones. EC cells accumulate 5-HT in secretory large, dense core vesicles (LDCVs), via vesicular monoamine transporter 1 (VMAT1), which transports monoamines from the cytosol into LDCVs. The biogenic amines are secreted through regulated exocytosis. The serotonin released from the EC cell is a transmitter regulating intestinal motility and transport in paracrine/endocrine and neuronal ways (Schafermeyer et al. 2004).

Two closely related isoforms of human vesicular monoamine transporters (VMATs) have been described and named: VMAT1 and VMAT2 (Erickson et al. 1996). They are, together with the vesicular acetylcholine transporter (VAcChT), members of the vesicular amine transporters (VATs) protein family. The VATs are expressed as integral proteins in the lipid bilayer membrane of secretory vesicles, where they transport biogenic amines and acetylcholine respectively. Transport of positively charged amines by members of the VATs family utilizes an electrochemical gradient across the vesicular membrane that is established by proton pumping. Protons are accumulated in the vesicles via a vacuolar ATPase not physically associated with the transporter (Parsons 2000). VMAT1 and VMAT2 have distinct tissue distribution and pharmacologic properties; VMAT1 expression is confined to EC cells of the gut and adrenal medulla and VMAT2 expression to neuronal cell populations and adrenal medulla (Erickson et al. 1996, Erickson & Varoqui 2000, Krantz et al. 2000, Jakobsen et al. 2001).

Secretory granules are specialized intracellular organelles that serve as a storage pool for selected secretory products. LDCVs are the functional equivalent of the secretory granules in which endocrine and neuroendocrine cells store peptide hormones. Sorting and storage during secretory granule biogenesis have been investigated for a long time but are not fully elucidated yet. Today we know that the initial assembly begins in the endoplasmic reticulum (ER), which is the common point of entry for newly synthesized exportable proteins. The next step in this pathway is entrance into the Golgi and trans-Golgi network. Whether exit from the ER is rate limiting or not is not clear yet. However, it is certain that delivery of different proteins to different

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destinations involves processes collectively known as protein sorting and delivering. During this process, membrane proteins of secretory granules, such as VMATs, require sorting signals for granule membrane targeting. The best evidence for sorting signals on granule membrane proteins comes from studies of a few model proteins in which the important signal is localized in the cytoplasmic C-terminus part of the proteins (Arvan & Castle 1998).

The predicted secondary structure of VMAT1, as for the other VATs proteins, reveals 12 transmembrane domains (TMD) with both the N-terminus and the C-terminus parts in the cytoplasm (Erickson et al. 1992, 1996, Liu et al. 1992, Eiden et al. 2004). VMAT1 contains aspartate (D) residues in TMDs 1, 10 and 11 that are thought to be critical for substrate recognition and transport (Eiden et al. 2004). The cytoplasmic C-terminus of all VATs contain sequences required for potentially phosphorylation-dependent trafficking to the correct vesicles; that is, synaptic-like microvesicles (SLMVs) for VACHt and LDCVs for VMATs. These sequences may also be important for recycling of the proteins via endocytosis and for phosphorylation that may control other aspects of the proteins (Liu et al. 1999). VACHt contains a C-terminal, cytoplasmic dileucine (LL) motif, while VMATs contain a dileucine-like (IL) motif for internalization into plasma membrane (Liu et al. 1999). VACHt contains serine (S) five residues upstream of the dileucine motif, while VMATs contain glutamate (E). These residue motifs appear to be of major importance to localize the protein in the proper vesicle (Krantz et al. 2000).

Classical midgut carcinoids are rare tumors arising from the ECs in the mucosa of jejunum, ileum, cecum and ascending colon of the gastrointestinal (GI) tract. The clinical features are related to secretion of humoral factors, such as serotonin, tachykinins, bradykinins and prostaglandins (Oberg 2002, 2003, Oberg et al. 2004a,b). Classical midgut serotonin-producing carcinoid tumors express predominantly VMAT1, while endocrine histamine-producing tumors almost exclusively express VMAT2 (Jakobsen et al. 2001). We have previously investigated VMAT1 gene expression and tissue distribution in midgut carcinoid specimens and in normal tissues (Vikman et al. 2005). During this analysis, we identified a novel shorter splicing variant of the VMAT1 gene that lacks exon 15 and that we now denote VMAT1A15. The number of nucleotides in exon 15 is not an even multiple of three. As a consequence, there is a shift of reading frame, and exon 16 (the last coding exon) is translated in an alternative reading frame, yielding a shorter and unrelated C-terminus of VMAT1A15 compared with the native VMAT1 protein. Here we show that VMAT1A15 appears to have a different cellular function from VMAT1, probably as a result of its different structure, conformation and cellular localization.

Materials and methods

Chemicals

The chemicals and suppliers used were as follows: PIPES, L-ascorbic acid, MgATP, pargyline hydrochloride, 5-HT, BSA, potassium tartrate, CHAPS, DTT and EDTA (Sigma); Digitonin (Serva, Heidelberg, Germany); CaCl2 and glucose monohydrate (VWR International, Stockholm, Sweden); and [3H]5-HT (PerkinElmer, Boston, MA, USA).

Cell culture

The human endocrine pancreatic tumor cell line BON, a generous gift from Prof. J C Thompson, Galveston, TX, USA, was cultured in DMEM (Dulbecco’s MEM with Glutamax-I) and F12 K nutrient mixture (Kaighn’s modification) at a 1:1 ratio, supplemented with 10% fetal bovine serum (FBS) and 1 mM sodium pyruvate. The monkey kidney cell line CV-1, a generous gift from Dr J Ericsson, LICR, Uppsala, Sweden, was cultured in DMEM, supplemented with 10% FBS, 1 mM sodium pyruvate and 0·1 mM nonessential amino acids. All cell culture reagents were from Invitrogen.

Tumor specimens

Tumor specimens from 15 patients diagnosed with midgut carcinoid tumor at the Department of Endocrine Oncology, University Hospital, Uppsala, Sweden, were included in the study. From two patients, we had both primary tumors and metastases, obtained at different times, while the rest of the primary and metastasis specimens were from independent patients. The study was approved by the local ethics committee (Dnr Ups 02–077).

Reverse transcription (RT)–PCR

Total RNAs were isolated from BON cells and flash-frozen midgut carcinoid specimens with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. An amount of 2 µg of each RNA sample was subjected to cDNA synthesis with Superscript RNase H– reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. PCR was performed on cDNA from BON cells, 15 midgut carcinoid specimens (10 primary tumors and 5 metastases) and commercially available cDNA from normal tissues (Clontech). PCR amplification was performed for 35 cycles with VMAT1 forward 5′-GGC ACC TGG TGG ATC TAC GCC ACA-3′ (exon 14) and VMAT1 reverse 5′-TGC GGC ACC AAG GCA TAG AGG AAG A-3′ (exon 16) primers. PCR products were analyzed on ethidium bromide (EtBr)-stained 1% agarose gels.
Quantitative real-time PCR

Total RNAs from 12 midgut carcinoid specimens (6 primary tumors and 6 metastases) and BON cells were extracted and reverse transcribed as described above. The cDNAs were analyzed for the expression of VMAT1 and VMAT1Δ15. Gene-specific PCR products were continuously measured by the iCycler IQ real-time detection system (Bio-Rad) during 40 cycles with iQ SYBR Green Supermix (Bio-Rad). The following primers were used in the study: Q-RT VMAT1/Δ15 forward 5'-GGT GGA TTC TTC TAT GAT GCC C-3' (exon 14), Q-RT VMAT1 reverse 5'-GTG GAT GGA CCT ATA GCA AAG C-3' (exon 15/ exon 16 boundary), Q-RT VMAT1Δ15 reverse 5'-CTC AGA ATA GCC TAT AGC AAA GC-3' (exon 14/ exon 16 boundary), Q-RT GAPDH forward 5'-CCC ATG TTC TCT CGA GCT ACT CCT CAT GGT CAG GCT-3' and Q-RT GAPDH reverse 5'-TGG TCA TGA GTC CTT CCA CGA TA-3'. The data were evaluated by the 2^ΔΔCT method (Livak & Schmittgen 2001), using the mRNA level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) from each individual sample for normalization, that is,

\[ \Delta \Delta C_T = (C_T, \text{VMAT1}_\Delta15 - C_T, \text{GAPDH}) - (C_T, \text{VMAT1} - C_T, \text{GAPDH}). \]

The plotted data represent the ratio between VMAT1Δ15 and VMAT1; that is, the percentage of VMAT1Δ15 mRNA expression in relation to VMAT1 mRNA expression.

Plasmid constructions

The following primers were used to amplify the entire coding sequences of VMAT1 and VMAT1Δ15: V-cloning forward 5'-GGC CAG ATC TAT GCT CCG GAC CAT TCT GGA-3' and V-cloning reverse 5'-CCT TCT CGA GCT ACT CCT CAT GGT CAG GCT-3'. PCR amplification was performed with Expand High Fidelity Enzyme (Roche). The thermocycling protocol was as follows: initial denaturation at 94 °C for 3 min followed by 10 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 2 min. Then, 15 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and elongation at 72 °C for 2 min with 15 s added to the elongation time for each cycle. The final extension was at 72 °C for 7 min. The two PCR products were isolated from a low melting point agarose gel, purified with the QIAquick Extraction Kit (Qiagen) and ligated into pCR2·1 (Invitrogen) by means of T/A cloning. The VMAT1 and VMAT1Δ15 coding sequences were then subcloned into pCDNA3 (Invitrogen) for in vitro transcription of VMAT1 and VMAT1Δ15 mRNAs. They were also subcloned into pEGFP-C2 (Clontech) to produce EGFP-VMAT1 and EGFP-VMAT1Δ15 fusion proteins with the EGFP sequence at the N-terminus. All constructs were sequenced and verified on an automated capillary sequencer.

In vitro transcription-coupled translation

35S-Met-labeled VMAT1 and VMAT1Δ15 proteins were produced by in vitro transcription-coupled translation from pCDNA3-VMAT1 and pCDNA3-VMAT1Δ15 with the TnT Quick Coupled Transcription/Translation Systems (Promega) and Redivue L 35S-methionine (Amersham) according to manufacturers’ protocols. Samples were resolved by electrophoresis on 12% SDS-PAGE. The gel was dried and subjected to autoradiography.

Western blot

BON cells were homogenized in ice-cold lysis buffer (10 mM potassium phosphate buffer, pH 6.8, containing 1 mM EDTA, 10 mM CHAPS, 1 mM DTT and complete Mini protease inhibitor cocktail tablets (Roche)). Homogenates were sonicated twice for 20 s and cleared by centrifugation for 10 min at 10 000 g. The supernatants were assayed for protein concentration with Coomassie Plus protein assay reagent (Pierce, Erembodegen, Belgium). Aliquots of 50 µg were resolved by SDS–PAGE, and transferred to nitrocellulose membranes (Bio-Rad). The BenchMark Prestained Protein Ladder (Invitrogen) was used to calculate the apparent size of proteins. The membranes were incubated with the rabbit polyclonal anti-VMAT1 antibody (H-100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 48 h, washed and incubated with the horseradish peroxidase (HRP)-coupled anti-mouse/rabbit IgG-POD antibodies (Roche). After being washed, the blots were visualized with the western blotting chemiluminescence luminol reagent (Roche). The membranes were stripped and reprobed with goat polyclonal anti-actin (I-19; Santa Cruz Biotechnology), washed and incubated with HRP-coupled donkey antigoat IgG (sc-2020; Santa Cruz Biotechnology). The blots were visualized with Western blotting chemiluminescence luminol reagent (Santa Cruz Biotechnology).

Immunoprecipitation

Aliquots of 1 mg total protein lysates from BON cells were incubated with 2 µg rabbit polyclonal anti-VMAT1 antibody (H-100; Santa Cruz Biotechnology) for 90 min on end-over-end at 4 °C. The antibody was raised against N-terminus peptides of the VMAT1 protein and should in theory be able to detect both VMAT1 and VMAT1Δ15. Then, 30 µl protein G Sepharose (Amersham) were added and the incubation continued for 60 min. Immunocomplexes were washed four times with RIPA buffer (10 mM Tris–HCL (pH 7.5), 150 mM...
NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, 0.1% SDS and complete Mini protease inhibitor cocktail tablets (Roche), resolved by 8% SDS–PAGE and transferred to nitrocellulose membranes (Bio-Rad). The BenchMark Prestained Protein Ladder (Invitrogen) was used to calculate the apparent size of proteins. The membranes were incubated for 48 h with the primary goat polyclonal anti-VMAT1 antibody (N19; Santa Cruz Biotechnology). The secondary antibody was HRP-coupled donkey antigoat IgG (Santa Cruz Biotechnology). The antigoat antibody cannot detect heavy and light chains of the rabbit antibody used for immunoprecipitation. The results were visualized with Western blotting chemiluminescence luminal reagent (Santa Cruz Biotechnology).

Transfection, immunofluorescence and confocal microscopy analyses

BON cells were plated on microscope glass cover slips in six-well plates, transfected with pEGFP-C2, pEGFP-VMAT1 and pEGFP-VMAT1Δ15 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were cultured for 24 h before analysis. For immunofluorescence analyses, the transfected cells on cover slips were washed in PBS and fixed in buffered 4% paraformaldehyde for 20 min at RT. The cover slips were mounted on glass object slides with Fluoromont G (Immunkemi, Stockholm, Sweden). Cells were photographed with a Hamamatsu ORCA CCD digital camera, using the QED Imaging System software with a Zeiss Axioplan2 microscope. For confocal microscopy analyses, the transfected cells on cover slips were washed in PBS, fixed and permeabilized in 0.2% Triton-X100 in PBS. They were then washed in PBS and incubated in 10 mM glycine in PBS. Primary and secondary antibodies were diluted in PBS containing 5% calf serum. Cells were incubated with a primary rabbit anti-human chromogranin A antibody (DAKO, Copenhagen, Denmark) followed by a secondary TRITC-conjugated antirabbit antibody (DAKO) with three PBS washing steps between. The cover slips were mounted on glass object slides with Fluoromont G. A Zeiss 510 META instrument (Zeiss, AB, Stockholm, Sweden) with Axiolab 200 microscope stand was used for all VMAT1 and VMAT1Δ15 colocalization studies. DAPI or Hoechst 33342 was exited with 405 nm, GFP with 488 nm and TRITC with 534 nm laser lines. Emission was detected by appropriate band-pass filters. Endoplasmic reticulum analyses were performed on live BON cells with 100 nM ER-Tracker Blue-White DPX (E-12353; Molecular Probes, Eugene, OR, USA), a photo stable probe that is selective for the ER in live cells. Nontransfected and transfected cells were then grown and stained on cover slips placed in medium in a POC-R cultivation chamber (Zeiss). All images were acquired at 63 magnification. For each image, a Z-series was acquired in multitracking mode, and images are presented as single projections. Colocalization between EGFP-VMAT1 or EGFP-VMAT1Δ15 and chromogranin A or ER was measured with the colocalization module of the Zeiss LSM 510 software.

[^5]H5-HT Uptake in permeabilized CV-1 cells

5-HT uptake was analyzed by a protocol similar to the one published by Erickson et al. (1992). CV-1 cells were transfected with pEGFP-VMAT1 and pEGFP-VMAT1Δ15. Transfection was performed as described above for BON cells. Cells were cultured for 24 h before analysis. Transfection efficiency was evaluated on a FACS Calibur (Becton Dickinson, San Diego, CA, USA), and at least 20,000 events were collected for each sample. Cells were then rinsed once with intracellular buffer (20 mM PIPES (pH 6.8) containing 110 mM potassium tartrate, 5 mM glucose, 0.2% bovine serum albumin, 200 µM CaCl2, 1 mM ascorbic acid and 10 µM pargyline). Thereafter, cells were permeabilized in intracellular buffer with 10 µM digitonin for 10 min at 37 °C. After permeabilization, the medium was replaced with intracellular buffer containing either 5 mM MgATP and [^3]H5-HT (0.1 µM, 111 kBq/ml) or 5 mM MgATP, [^3]H5-HT (0.1 µM, 111 kBq/ml) and 5-HT (100 µM). Cells were incubated for 5 min at 37 °C. The uptake was terminated by washing cells with 1 ml cold intracellular buffer and with an additional 1 ml cold PBS. Cells were then trypsinized and counted (Z2; Beckman Coulter Counter, Fullerton, CA, USA), and cell-associated [^3]H5-HT was measured in a liquid scintillation counter (1214 RackBeta Excel, LKB-Wallac, Upplands Vasby, Sweden) with Quicksafe A (Zinsser Analytic, Uppsala, Sweden). All the experiments were performed in triplicates. Transfection efficiency was evaluated by direct detection of EGFP on FACS Calibur, and at least 20,000 events were collected for each sample.

Results

Identification of an alternatively spliced VMAT1 sequence

To clone the full-length coding sequence of VMAT1, we amplified it by PCR from cDNA prepared from BON cells with VMAT1-specific cloning primers. To our surprise, we identified two distinct PCR products on the agarose gel, which were T/A-cloned and sequenced. One product was, as expected, the already known VMAT1 coding sequence, comprising 1575 bp from 16 exons. The second product was shorter, with the first 1330 bp identical to the 5’ coding sequence of VMAT1.
and the last 111 bp identical to the 3’ coding sequence of VMAT1. By analysis of the transcript, the genomic sequence organization and exon/intron boundaries, we concluded that the novel sequence is produced by alternative splicing of VMAT1 where the next to last exon, exon 15, is excluded. We therefore named the novel sequence VMAT1Δ15. The number of nucleotides in exon 15 is not an even multiple of three. As a consequence, there is a shift of reading frame, and exon 16 is translated in an alternative reading frame (Fig. 1A). This means that the putative novel VMAT1Δ15 protein has a different and shorter C-terminus than that of the native VMAT1 protein (Fig. 1B). The total number of amino acids for VMAT1 is 525, but it is 472 for VMAT1Δ15. The sequences diverge at the end of transmembrane domain 11 of VMAT1, and the VMAT1Δ15 protein lacks the dileucine-like motif that is important for proper trafficking to secretory vesicles. Online prediction analyses of transmembrane helices for VMAT1 and VMAT1Δ15 (www.cbs.dtu.dk/services/TMHMM/) reveal that VMAT1 has 12 transmembrane domains with both the N-terminus and the C-terminus in the cytoplasm, while VMAT1Δ15 has 11 transmembrane domains with the N-terminus in the cytoplasm. The smaller size of VMAT1Δ1 and the lack of a vesicular targeting sequence led us to hypothesize that VMAT1Δ15 has a different conformation and possibly a different cellular localization from VMAT1 (Fig. 1C).

**VMAT1Δ15 gene expression in BON cells, midgut carcinoids and normal tissues**

After having identified the VMAT1Δ15 transcript in BON cells, we wanted to determine whether the transcript is expressed also in normal cells. We therefore analyzed cDNA from normal GI-tract tissues, which are known to express VMAT1, using PCR primers designed in exons 14 and 16 of VMAT1. We found that VMAT1 and VMAT1Δ15 are simultaneously expressed in these tissues with an apparently higher expression of VMAT1 relative to VMAT1Δ15 (Fig. 2A). We next analyzed the differential expression of VMAT1 and VMAT1Δ15 in midgut carcinoid specimens and BON cells, and found a simultaneous expression profile (Fig. 2B). Since VMAT1 expression appears to be significantly higher than VMAT1Δ15 expression in all tissues studied, we went on to perform quantitative real-time RT–PCR analysis. We analyzed six primary midgut carcinoid tumors, six midgut carcinoid metastases and BON cells. The relative expression of VMAT1Δ15 in relation to VMAT1 varied from 4·2% to 22·8% among the carcinoid specimens as evaluated by the 2−ΔΔCt method (Fig. 3). No general trend in the ratio was observed when comparing primary tumors (Fig. 3A) with metastases (Fig. 3B). From this analysis, we could not determine whether their relative expression is important for the malignant transformation of neuroendocrine cells. However, in two cases (C12 and C16) where primary tumors and metastases were obtained from the same patients, it was apparent that VMAT1Δ15 expression in relation to VMAT1 expression is higher in the metastases than in the primary tumors.

**VMAT1 and VMAT1Δ15 protein expression**

After having characterized the VMAT1Δ15 transcript, we verified that the novel VMAT1Δ15 transcript could be translated into a protein of expected size by in vitro transcription-coupled translation (Fig. 4A). Thereafter, we set out to detect the endogenous VMAT1Δ15 protein. A commercial antibody raised against an epitope in the N-terminus part of the protein, shared by VMAT1 and VMAT1Δ15, detected only one band in total lysate from BON cells by western blot analyses, presumably the native form of VMAT1, but failed to detect the smaller VMAT1Δ15 protein (Fig. 4B). However, when combining immunoprecipitation with western blotting, starting from as much as 1–2 mg proteins from total lysate of BON cells, we finally managed to detect two bands, presumably VMAT1 and VMAT1Δ15 (Fig. 4C). In this case, a rabbit polyclonal anti-VMAT1 antibody was used for immunoprecipitation, and a goat polyclonal anti-VMAT1 antibody was used for detection.

**VMAT1Δ15 is localized not in vesicles but in the ER of transfected BON cells**

Considering that the LDCV targeting sequence at the C-terminus part of VMAT1, with the dileucine-like motif and two glutamate residues in positions −4 and −5, is absent in VMAT1Δ15, we proceeded to investigate the cellular localization of VMAT1Δ15. In order to do so, BON cells were transiently transfected with pEGFP-C2, pEGFP-VMAT1 or pEGFP-VMAT1Δ15. The expressed fusion proteins were evaluated by Western blot analysis, resulting in the expected sizes (data not shown). Immunofluorescence microscopy revealed that EGFP-VMAT1 stained LDCV-like structures (Fig. 5A). EGFP was unspecifically located in the cytoplasm of the transfected cells, showing that the EGFP tag did not interfere with the normal protein localization.

We next investigated whether EGFP-VMAT1 and EGFP-VMAT1Δ15 were colocalizing with the vesicular marker chromogranin A. The confocal microscopy analysis results shown in Fig. 5B illustrate the distribution of chromogranin A (red) and either EGFP-VMAT1 (green) or EGFP-VMAT1Δ15 (green). As expected, the distribution of chromogranin A was...
A

**VMAT1 genomic sequence**

Exon 14  Intron  Exon 15  Intron  Exon 16

...GCTATAG GTAT.....CCAG GTCCATCC.....GAGAAGCTT GTAA.....GCAG GCTATTCTG...

**VMAT1 mRNA / protein**

```
-- GCT ATA G
      A I
-- GT CCA TCC
      G P S
      E K L
-- GAG AAG CTT
      A I L
```

**VMAT1Δ15 mRNA / protein**

```
-- GCT ATA G
      A I
-- GC TAT TCT
      G Y S
```

B

**VMAT1**

```
-- AIGPSXGGA1VKAIGFPWLMVITGVINIVAPLCCYLYRSPPAKGKLAIL5QOCMPETRMATQKPTKFGDSDSDDEPDHEE
```

**VMAT1Δ15**

```
-- AIGYESGLPGRDPDPVCNPEAHEGIGSSGGQ
```

C

**VESICLE LUMEN**

**VMAT1Δ15**

C-terminus

**CYTOPLASM**

VMAT1

C-terminus
dileucine-like motif
naturally granular, as was generally the EGFP-VMAT1 distribution, with a partial overlap when the two images were merged (yellow). For EGFP-VMAT1/afii979715, the degree of colocalization was significantly lower, and the expression pattern again appeared to be associated with cytoplasmic membrane structures, indicating ER targeting. This was taken as a further proof of different cellular localization of the two proteins. Extended analyses of subcellular localization were performed on transfected cells with fluorescent molecules targeting intracellular organelles. Staining of the ER (red) in combination with EGFP-VMAT1 (green) expression revealed few areas of yellow, indicating poor colocalization, while for EGFP-VMAT1/afii979715 (green) there were extensive yellow areas, indicating substantial colocalization. Furthermore, neither EGFP-VMAT1 nor EGFP-VMAT1/afii979715 colocalized with Golgi staining (data not shown).

**VMAT1-transfected, but not VMAT1Δ15-transfected, CV-1 can take up 5-HT**

Considering the different conformation and distribution of VMAT1Δ15 and VMAT1, we next investigated the serotonin (5-HT) uptake capacity, that is, the transporting capacity, of the two proteins in digitonin-permeabilized CV-1 cells. We measured and compared [3H]5-HT uptake in pEGFP-VMAT1-transfected, pEGFP-VMAT1Δ15-transfected and nontransfected CV-1 cells. Transfection efficiencies were evaluated by flow cytometry, yielding 30% EGFP-VMAT1-positive cells and 26% EGFP-VMAT1Δ15-positive cells (Fig. 6A). The results from the [3H]5-HT uptake studies, corrected for transfection efficiency, are shown in Fig. 6B. Specific [3H]5-HT uptake was obtained by the EGFP-VMAT1-transfected CV-1 cells, since the uptake was readily

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**Figure 1** VMAT1Δ15 is an alternatively spliced form of VMAT1. (A) During RNA splicing of VMAT1, exon 15, the second to last exon, can sometimes be spliced out. This alternatively spliced variant of VMAT1 was denoted VMAT1Δ15. The number of nucleotides in exon 15 is not an even multiple of three. As a consequence, there is a shift of reading frame, and exon 16 of VMAT1Δ15 is translated in an alternative reading frame from VMAT1. (B) The alternative translation of exon 16 yields a VMAT1Δ15 protein with a shorter C-terminus sequence that is unrelated to the sequence of VMAT1. (C) The C-terminus of VMAT1Δ15 diverges from VMAT1 at the end of TMD 11. Since it is short and not hydrophobic, it may remain in the vesicle lumen, if the VMAT1Δ15 protein is targeted to the membrane of an intracellular LDCV, as VMAT1 is supposed to be. In addition, VMAT1Δ15 lacks the dileucine-like motif that is believed to target VMAT1 to LDCVs.

**Figure 2** VMAT1Δ15 is coexpressed with VMAT1 in midgut carcinoid specimens and normal GI tract. (A) PCR analyses were performed on normal GI-tract tissues, using commercially available cDNA. (B) RT–PCR analyses were performed on mRNA from 15 midgut carcinoid specimens (10 primary tumors and 5 metastases) and BON cells. The PCRs were performed with a VMAT1-specific forward primer located in exon 14 and a reverse primer located in exon 16. The obtained VMAT1 PCR product is 539 bp, covering part of exon 14, exon 15 and part of exon 16, while the VMAT1Δ15 PCR product is 400 bp, covering part of exons 14 and 16. The obtained PCR products were visualized by EtBr-stained agarose gel electrophoresis.
competed by a 1000 molar excess of nonlabeled 5-HT. No uptake of \[^3H\]5-HT was obtained by the EGFP-VMAT1\_afii979715-transfected cells. As expected, CV-1 cells were not able to take up \[^3H\]5-HT since they do not express VMAT1.

**Discussion**

VMAT1 is an integral protein in the membrane of LDCVs of neuroendocrine and endocrine cells that allows the transport of biogenic monoamines, such as serotonin, from the cytoplasm to the vesicles (Erickson & Varoqui 2000, Eiden *et al.* 2004). The VMAT1 gene is located on chromosome 8 (NCBI gi 22046760), and the full-length VMAT1 transcript is produced from 16 exons (NCBI U39905, gi 1314289). The transcript encodes a protein of 56 kDa that can be post-transcriptionally modified by glycosylation and phosphorylation (Erickson & Varoqui 2000). We identified an alternative splicing variant of VMAT1 during amplification of the full-length coding sequence. Upon DNA sequencing, BLAST analysis and analysis of the exon/intron boundary regions, we found that the alternatively spliced form of VMAT1 lacks exon 15. It was therefore denoted VMAT1\_afii979715. We showed that VMAT1 and VMAT1\_afii979715 have similar gene expression profile in normal tissues of the GI tract and carcinoid tumors, and that VMAT1 expression in all cases is higher than VMAT1\_afii979715 expression. Whether the relative expression of the two forms is changed during the malignant transformation requires a more detailed analysis, using a larger number of primary tumors and metastases from the same patients. However, analysis of the primary tumors and metastases from two patients shows that the relative expression changes with a relative increase in VMAT1\_afii979715 expression during carcinoid progression.

The physical description of VMAT1 predicts that it has 12 transmembrane domains and that the N-terminus and C-terminus parts are cytoplasmic (Eiden *et al.* 2004). The cytoplasmic C-terminus of the other VAT proteins, that is, VAChT and VMAT2, have been shown to contain sequences required for localizing the proteins to the correct vesicle type (Varoqui & Erickson 1998a,b, Erickson & Varoqui 2000). In PC12 cells, the VAChT localizes preferentially to SLMVs, whereas the closely related VMAT2 localizes preferentially to LDCVs (Liu *et al.* 1994, Liu & Edwards 1997). VMAT1 contains a homologous motif to VMAT2 in the C-terminus part of the protein, consisting of a dileucine-like motif with two glutamate residues in positions –4 and –5. This short sequence is needed for protein trafficking and localization (Liu *et al.* 1999, Krantz *et al.* 2000). The C-terminus part of the protein predicted to be encoded by the VMAT1\_afii979715 transcript does not contain this sequence and is therefore unable to target LDCVs. By using EGFP-fusion proteins, we show that EGFP-VMAT1 is, as expected, located in secretory vesicles with a partial colocalization with the neuroendocrine secretory vesicle marker chromogranin A (Kim *et al.* 2001). EGFP-VMAT1\_afii979715 did not colocalize with chromogranin A but showed a high degree of colocalization with the ER. The retention of VMAT1\_afii979715 in the ER may simply be the consequence of a lack of a vesicle target signal, since VMAT1\_afii979715 lacks the classical ER retention signal.
Furthermore, VMAT1Δ15 does not contain any of the motifs lately recognized for ER targeting, such as AIAKE or HIEL (Wrzeszczynski & Rost 2004, Gilham et al. 2005). The regulation of monoamine transporter activity has a pivotal role in the physiology of normal and cancer cells. The transport properties of VMAT1 and VMAT2 have previously been determined in transfected digitonin-permeabilized CV-1 cells (Erickson et al. 1996). The ability of these cells to support transport of monoamines and the energetic requirements of this process shows that these transporters are targeted to intracellular compartments that contain an electrogenic vacuolar type H+ pump (Erickson et al. 1996). Since we had already shown that VMAT1Δ15 is not able to target the same vesicles as VMAT1, we wanted to investigate whether VMAT1Δ15 could take up serotonin. We studied [3H]5-HT uptake capacity in digitonin-permeabilized CV-1 cells transiently transfected to express either EGFP-VMAT1 or EGFP-VMAT1Δ15. We confirmed the ability of VMAT1 to take up [3H]5-HT and showed that VMAT1Δ15 is unable to take up [3H]5-HT. We believe that the different capacities in serotonin uptake are due to the different C-terminus of the proteins, which leads to targeting of different cellular compartments. The importance of vesicle proteins in the diagnosis and treatment of neuroendocrine tumors has been discussed, and recently it was demonstrated that VMAT1 is important for the uptake of 123I-MIBG in tumor cells (Kolby et al. 2003, Nilsson et al. 2004). This compound has an important role in scintigraphy and radionuclide therapy of neuroendocrine tumors, but whether a relative increase of

**Figure 4** VMAT1 and VMAT1Δ15 protein expression. (A) In vitro transcription-coupled translation analysis of 35S-Met-labeled VMAT1 and VMAT1Δ15. Size markers in kDa are indicated on the left. (B) Western blot analysis using 50 µg total protein lysate from BON cells and a rabbit polyclonal antihuman VMAT1 antibody (H-100). Size markers in kDa are indicated on the left. (C) Immunoprecipitation analysis using 1 mg total protein lysate from BON cells and a rabbit polyclonal antihuman VMAT1 antibody (H-100). The immunocomplexes were denatured, electrophoresed and blotted. VMAT1 and VMAT1Δ15 were then detected with a goat polyclonal anti-VMAT1 antibody (N19). Size markers in kDa are indicated on the left.
A  EGFP-VMAT1  EGFP-VMAT1Δ15  EGFP

B  Chromogranin A  EGFP-VMAT1  Merge

Chromogranin A  EGFP-VMAT1Δ15  Merge

C  ER  EGFP-VMAT1  Merge

ER  EGFP-VMAT1Δ15  Merge
Figure 6 VMAT1, but not VMAT1Δ15, takes up serotonin. CV-1 cells were transiently transfected with EGFP-VMAT1 or EGFP-VMAT1Δ15. (A) Cells from some wells were analyzed by flow cytometry to determine the transfection efficiency. (B) Some cells were permeabilized with digitonin and assayed for their ability to take up tritium-labeled serotonin ([3H]5-HT). A 1000 molar excess of nonlabeled 5-HT was used to compete specifically with the transport of [3H]5-HT. Average uptake of [3H]5-HT from triplicate wells is given in attomol per cell for 5-HT competed (open bars) and not competed (filled bars) cells. Error bars are maximum errors.

Figure 5 Cellular localization of VMAT1 and VMAT1Δ15. (A) Immunofluorescence microscopy analysis of BON cells transiently transfected with EGFP-VMAT1, EGFP-VMAT1Δ15 or EGFP. (B) Confocal immunofluorescence microscopy analyses of BON cells transiently transfected with either EGFP-VMAT1 (green) or EGFP-VMAT1Δ15 (green) and stained with an anti-chromogranin A antibody (red). The merged image shows the degree of colocalization (yellow). Arrowheads indicate yellow areas. One representative set of images is shown. (C) Confocal immunofluorescence microscopy analyses of live BON cells transiently transfected with EGFP-VMAT1 (green) or EGFP-VMAT1Δ15 (green) and incubated with a fluorescent ER marker (red). The merged image shows the degree of colocalization (yellow). One representative set of images is shown.
VMAT1Δ15 expression in relation to VMAT1 affects this uptake is not known.

Previous studies have demonstrated the biologic relevance of alternative splicing in health and disease. Although alternative splicing of different genes has been considered to be associated with different stages of tumorigenesis, and splicing variants have been characterized as tumors markers, it is still not clear whether this is a common phenomenon or not (Kirschbaum-Slager et al. 2004). Recently, various studies have shown that different subcellular association of different splice isoforms depend on unique sequences present in tail anchors (Yao et al. 2004, van Herpen et al. 2005). A more detailed expression profile of the two VMAT1 isoforms in classical serotonin-producing midgut carcinoid tumors awaits the availability of isoform-specific antibodies.

In summary, we have identified and characterized a novel alternative splicing form of VMAT1, denoted VMAT1Δ15, which yields a novel protein with different localization and function from VMAT1. Further investigation is needed to determine whether the regulation of VMAT1 function is due to some kind of equilibrium between the two forms of VMAT1 in both normal and malignant tissue. This might be of importance to understand VMAT1 gene regulation and to develop new drugs.

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