FULL LENGTH CLONING OF THE SNARE PROTEIN VAMP IN THE SHARK (*SQUALUS ACANTHIAS*) RECTAL GLAND

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The Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is expressed in several shark tissues, including rectal gland, brain, intestine, testis and spleen (Hemminger et al., *Bull. MDIBL* 36:33-35, 1997). Recently, the soluble N-ethylmaleimide sensitive factor attachment protein receptor element (SNARE) protein syntaxin 1A was shown to inhibit CFTR currents by domain specific protein interactions (Naren et al., *PNAS* 95:10972-7, 1998). Preliminary data from the Frizzell laboratory demonstrates that co-expression of the SNARE protein VAMP-2 in A6 cells expressing CFTR results in a three-fold higher forskolin stimulated short-circuit current compared to controls (Peters et al., *Pediatric Pulmonology Supplement* 20, 2000). In the present study, we examined the hypothesis that SNARE proteins are expressed in the shark rectal gland (SRG). We report the full length cloning of a VAMP-like protein from this chloride secreting marine epithelium.

Total RNA was isolated from fresh dogfish rectal gland tissue using cesium chloride method (Molecular Cloning). RNA was subjected to a DNAse 1 digest and reverse transcription was carried out with oligo(dT) primers (Clontech Advantage™). Degenerate primers were designed from two highly conserved regions found in several mammalian VAMP proteins. The sense primer 5'-YTNCARCARACNCARGCNCAR-GT-3' and antisense primer: 5'- ITTYTTCCACCARTAYTT-3' were used to amplify a fragment of rectal gland VAMP. PCR was performed for 35 cycles (95 °C for 60 sec; 53 °C for 60 sec; 68 °C for 45 sec) using Expand™ Long DNA polymerase (Boehringer Mannheim). PCR products from rectal gland of the expected size (~ 200 bp) were cloned (TOPO-cloning, Invitrogen) and sequenced (MDIBL Marine DNA Sequencing Facility).

Figure 1 illustrates the strategy for cloning SRG VAMP. Figure 2A shows the PCR results from rectal gland using degenerate primers designed from mammalian VAMP protein sequence. A weak but distinct product was amplified from rectal gland cDNA at an annealing temperature of 53 degrees. This band was cloned and sequenced and the putative protein sequence had greatest homology to mouse VAMP-2 (93%). To obtain the full length sequence, SMART RACE-PCR was performed as described

(Clonetech SMART RACE cDNA Amplification Kit) and the products were subcloned by TOPO-TA cloning (Invitrogen). Clones were screened by PCR for proper insert size and were then sequenced.

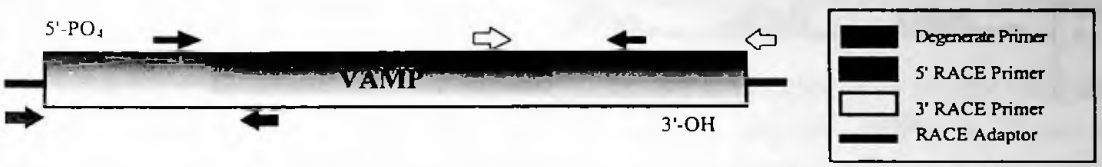

Figure 1: Schematic representation of VAMP cloning strategy. The black arrows indicate the location of degenerate primers. The adapters are represented as black bars located at transcript end. The gray and white arrows show the location of 3' RACE-PCR primers and 5' RACE-PCR primers, respectively.

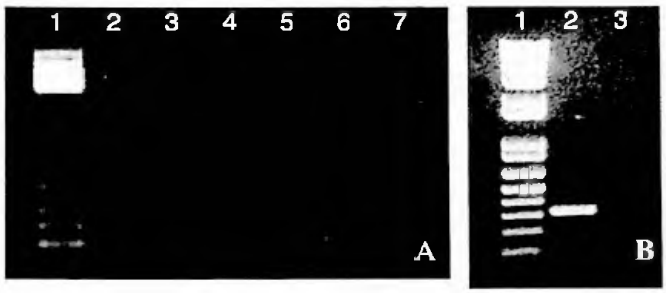

FIGURE 2: Gel electrophoresis of PCR products. Panel A: products from degenerate PCR using SRG cDNA: 123bp DNA ladder (lane 1), Gradient annealing temperature 43-53 degrees (lane 2-6), negative control with no cDNA (lane 7). A distinct band (~200bp) was obtained with the annealing temperature of 53 degrees (lane 4). Panel B displays a 318bp PCR product identified as full length VAMP: 1kb-plus DNA ladder (lane 1), a 318bp PCR product from shark rectal gland (lane 2), and a negative control (lane 3).

The full length construct of rectal gland VAMP was obtained using a gene specific sense primer originating at the start codon and antisense primer located the stop codon (Figure 2B). The full-length protein sequence of the shark VAMP had greatest similarity to human VAMP-3 (80% identity) using DNASTAR Molecular Biology software Megalign. Phylogenetic analysis of dogfish VAMP groups this clone with human and mouse VAMP-3 (data not shown). Rectal gland VAMP shares 68% identity with electric ray VAMP, 75% with mouse VAMP-3, and 63% with c. *elegans* VAMP (Figure 3). Based on hydrophilicity plot of the amino acid sequence we predict the membrane-anchoring region to be located at the C-terminus of this protein (Figure 4).

Percent Identity

1	2	3	4	5	6	7	8		
	64.2	69.8	76.7	75.0	74.8	73.3	86.0	1	Bovine VAMP
		63.2	62.4	66.0	63.1	60.6	63.3	2	C. elegans VAMP-1
			68.9	80.0	75.7	67.9	69.8	3	Dogfish Shark VAMP
				70.0	70.9	83.9	76.3	4	Electric Ray VAMP
					80.0	69.0	72.0	5	Human VAMP 3
						68.9	71.8	6	Mouse VAMP 3
							75.4	7	Rat VAMP 1
								8	Xenopus VAMP

Fig. 3: Percent identity between shark VAMP and VAMPs from other species was calculated by DNASTAR software. Percent homology of other species to shark is shown in bold.

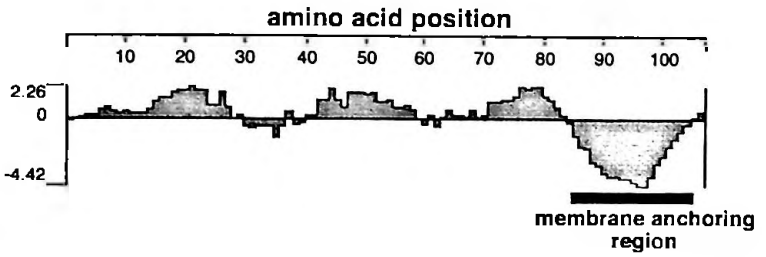

FIGURE 4: Hydrophilicity Plot of the shark rectal gland VAMP. The strongly hydrophobic region at the C-Terminus corresponds to the predicted membrane-anchoring region and is underscored with a black line. Horizontal scale represents the amino acid position.

In summary, we report the full length cloning of a shark rectal gland VAMP protein that has highest homology to VAMP-3 in higher vertebrates. We will focus further studies on the possible regulatory function of this protein in the membrane trafficking of CFTR.

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