Korean J Biol Sci 7: 133-137, 2003

Screening of cDNAs Encoding Secreted and Membrane Proteins in the Nervous System of Marine Snail *Aplysia kurodai*

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Key Words:
Aplysia
signal sequence trap
secreted protein
membrane protein
screening
yeast

Secreted proteins and membrane proteins play key roles in the formation, differentiation, and maintenance of multicellular organisms. In this study, we undertook to characterize these protein types in the central nervous system of the marine snail *Aplysia kurodai* using a yeast-based signal sequence trap method. One hundred and three cDNA clones were obtained by screening 300,000 clones from the signal sequence trap cDNA library. Of these, twelve were identical to previously identified *Aplysia* genes, 19 were related to known proteins in other organisms, and 54 clones were novel. These 54 new genes had high signal peptide scores or were found likely to contain a transmembrane domain sequence. Only 18 of the 103 clones proved to be false positive. The study demonstrates that the signal sequence trap method is an effective tool for Isolating *Aplysia* genes encoding secreted and membrane proteins.

Individual cells communicate with each other or with the environment by secreting and receiving signaling molecules, such as growth factors, hormones, neuropeptides and neurotransmitters. In addition to the secreted proteins and receptors required for such signaling, ion channels - a class of membrane proteins - play an essential role in determining the electrical properties of neurons and in the generation of electrical signals in neural circuits. These membrane proteins and secreted proteins contain a short amino-terminal signal peptide sequence (von Heijne, 1985; Kaiser and Botstein, 1987), which enables them to be targeted to the secretary pathway. The signal sequence trap method was developed from this observation.

The signal sequence trap method is a simple and effective tool for cloning secreted or membrane proteins. Tashiro et al. (1993) first developed the COS cell-based signal sequence trap system and this was further developed by Klein et al. (1996) into a yeast-based signal sequence trap system. Since then, many signal sequence trap screening results have been reported for various organisms using mammalian (Klein et al., 1996; Nakamura et al., 1995; Hamada et al., 1996), and plant systems (Kristoffersen et al., 1996; Goo et al., 1999b).

The yeast-based signal sequence trap makes use of the *S. cerevisiae* enzyme invertase, which plays a critical role under certain metabolic conditions. For example, if sucrose is provided as a carbon source instead of glucose, yeast must first secret invertase to catalyze the breakdown of the sucrose into glucose and fructose (Carlson et al., 1983). In the signal sequence trap system, mutant invertase lacking its initiator methionine and signal sequence is used to select for signal sequence of secreted and membrane proteins. For example, the cDNA cloning vector, pSMASH (Goo et al., 1999a), was used to carry a modified invertase gene lacking the leader sequence. The signal sequences and start codons of the secreted or membrane protein genes fused upstream of this defective invertase can provide the signals required to restore secretion, which would then allow the yeast to grow on sucrose-containing medium.

In this study, we applied the yeast-based signal sequence trap method to characterize the secreted and membrane proteins of the nervous system of the marine snail *Aplysia*, and isolated the genes encoding these proteins. Our results show that the yeast-based signal sequence trap method can be applied successfully to *Aplysia* neuronal genes.

Materials and Methods

Construction of pSMASH-5HT2c for testing the invertase selection

pSMASH-5HT2c contains an N-terminal sequence encoding from the initiation Met to the 4th transmembrane

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domain of mouse 5HT2c receptor cDNA. To obtain this sequence, a plasmid DNA pBS-5HT2c was amplified with two primers, 5HT2c-s and 5HT2c-T4 (5HT2c-s: 5-GGAAT-CCAAGCAATAATGGTGAACC-3; 5HT2c-T4: 5-AATATGC-GGCCGCTCACGAACACTTTGCTT-3). PCR was performed in a total volume of 50 µl containing 1X pfu DNA polymerase buffer, 200 µM each deoxynucleotide triphosphate, 2.5 units of cloned pfu DNA polymerase (Promega), 10 pmol of each primer, and 100 ng of plasmid DNA. Cycling conditions were 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min for 30 cycles, followed by a 5 min extension at 72°C. The PCR product and the pSMASH vector were digested with EcoRI and NotI, and the digested PCR product was inserted into pSMASH at the EcoRI and Not I sites. A pSMASH-5HT2c was then constructed by cloning a PCR-amplified cDNA into pSMASH.

Library construction

Poly (A)$^+$ RNA was isolated from *Aplysia kurodai* using oligotex dT30 (Roche Inc.). First strand cDNA was synthesized using a random primer and the superscript RNA isolation kit (Gentra Systems Inc.). The second strand was then synthesized and the double stranded cDNA produced was ligated with EcoRI linkers. cDNA fragments were digested with EcoRI, and ligated into the EcoRI site of the pSMASH vector.

Yeast transformation and selection

The library pSMASH DNAs were introduced into DBYα2445 (Matα, suc2Δ-9, lys2-801, ura3-52, ade2-101) by lithium acetate transformation, and the yeast cells were directly spread on sucrose plates (2% sucrose, 2% peptone, 1% yeast extract, 2% agar). Colonies appeared after incubating for 5-6 days at 30°C. The colonies were further cultured in sucrose media, and plasmid DNAs were isolated from the individual colonies. These plasmid DNAs were introduced into *E.coli* strain DH5α by heat-shock, and then purified and sequenced in both strands using forward and reverse primers with sequences corresponding to ADH1 promotor of pGAD424 (5-CTC GTT CCC TTT CTT CCT TGT TTC-3) and the suc2 gene (5-GGA CCA AAG GTC TAT CGC TAG TTT C-3).

DNA sequence analysis

The cDNA clones were sequenced using an automatic sequencer (ABI Prism, Perkin Elmer), analyzec with EditSeq, and translated using ExPASy (http://ca.expasy.org/tools/), and homology-searched with BLAST n the GenBank database. The presence of a signal peptice and of a transmembrane domain were predicted using the on-line programs, SignalP (http://www.cbs.dtu.dk/services/SignalP), PSORT II (http://psort.nibb.ac.jp), and SOSUI (http://sosui.proteome.bio. tuat.ac.jp).

Results

The signal sequence trap technique has been app ied to isolate secreted and membrane proteins with signal sequences. To determine whether a signal sequence trap

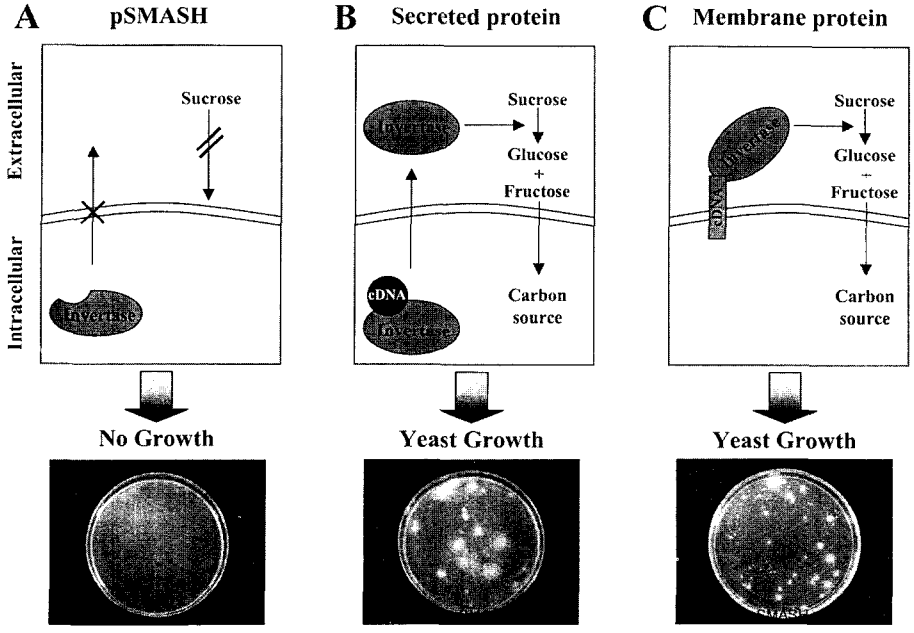

Fig. 1. Schematic representation of the signal sequence trap. Each panel shows a schematic diagram of the transformation of yeast strain DBYα2445, and of the ability of a yeast strain, lacking the invertase gene, and transformed with various DNA constructs to grow on sucrose medium. Result of transformation of a yeast strain lacking an invertase gene but containing a suc2 (invertase) gene, from which the signal peptide and the initiator methionine had been deleted (A), a mutant invertase fused to a secreted protein (B) and fused to a transmembrane domain from 5-HT2c (C).

Table 1. Summary of cDNA clones isolated from signal peptide selection libraries

	cDNAs sequenced	Unique gene classes
Identica to known proteins	12 (12%)	7 (11%)
Related to known proteins	19 (18%)	7 (11%)
Novel	54 (53%)	31 (50%)
S score>0.48	44	25
S score<0.48, containing transmem Brane domain	10	6
Others		
Total	18 (17%)	17 (28%)
	103	62

[a] Two clones are rRNAs, and 16 clones have no signal peptide sequence.

Table 2. cDNA clones with amino acid sequence identical to known *Aplysia* proteins

Clone	GenBank accession number	Protein name	Cellular location
1-2, 9, 11-4	M25886	Egg-laying hormone	S
18	U85585	*Aplysia* APGWamide	S
17, 110, 142	M13649	L5 neuropeptide precursor	S
41	A30330	Neuropeptide pep	S
123	AY033828	Neuropeptide CP2 precursor (CP2PP)	S
11-2	P12284	Neuroactive polyprotein R15-1 precursor	S
24, 11-3	M64342	*Aplysia* sp. neuropeptide	S

[a] S; secreted

can be used for identifying neural membrane proteins, the N-terminal sequence encoding from the initiation methionine to the 4th transmembrane domain of the mouse 5-HT2c receptor gene was inserted into the EcoRI-NotI site of pSMASH in frame with the invertase. In view of the topology of G-protein coupled receptors, it was expected that invertase fused to the C-terminal part of the 5-HT2c receptor would be exposed to the external matrix. In this manner, invertase would be able to catalyze the breakdown of sucrose into glucose and fructose, thus allowing the mutant yeast to survive in a medium containing sucrose instead of glucose as a carbon source. As shown in Figure 1, yeast transformation of pSMASH-5HT2c gave rise to 10^5 colonies per μg of DNA after 5 days of incubation on sucrose medium at 30°C. However, compared to the secreted proteins containing signal sequences, the growth rate of 5-HT2c-transformed yeast was slow (data not shown). In contrast, pSMASH vector alone did not form colonies on sucrose medium (Fig. 1). These results indicate that the transmembrane domain can function as a signal sequence. Therefore, the signal sequence trap method can be applied to effectively select membrane proteins that have no signal sequence.

Initially, a cDNA library was constructed in pSMASH using mRNA from the central nervous system of *Aplysia kurodai*. The cDNA library plasmids were then transformed into DBYα2445, containing a defective invertase gene, and colonies were obtained from sucrose medium after 5-6 days at 30°C. From 300,000 cfu of the cDNA library, 103 clones were found to be able to grow on sucrose medium. Plasmid DNA was prepared from these colonies and inserts were sequenced.

As shown in Table 1, 103 clones were obtained and further analyzed. Among these, 12% of the cDNAs were found to encode the known *Aplysia* genes, 18% were the related genes identified in other species, and 54% of the cDNAs were unknown genes. The other 17% were false positives. The seven unique cDNAs encoding known proteins are listed in Table 2. As shown in Table 2, all the clones encode neuropeptides or hormones. For example, egg-laying hormone is a well-known secretory protein of *Aplysia*. The other seven unique cDNAs, which shared homologies with known proteins, are listed in Table 3. These known proteins were identified as either secreted or membrane proteins.

The 31 novel genes are listed in Table 4. To determine whether these novel cDNAs are secreted or membrane proteins, their putative amino acid sequences were examined for the presence of a signal peptide with a signalP program. An S score of above 0.48 is indicative of the presence of a putative signal sequence. As shown in Table 4, 25 of 31 novel clones fell into this category. In addition, the six clones with S scores below 0.48 had amino acid sequences that were deemed likely to contain a transmembrane domain. Eighteen of the other clones were mis-selected, and had S scores lower than 0.48 and no putative transmembrane domain.

Table 3. cDNA clones with amino acid sequence homologous to known proteins

Clone	GenBank accession number	Protein name	E value	Cellular location	Function
3-13, 3-22	AAM22783	Neurocan	4e-06	S	Inhibition of neuronal adhesion and neurite outgrowth
13, 28, 40, 135, 104, 107, 137, 125, 11-7, 124	AAB48564	Neuropeptide GFAD precursor	3e-09	S	Neuropeptide
39, 115	AAB41699	Neuroendocrine protein 7B2	6e-33	S	Neuroendocrine
126	AAN61407	Matrilin	1e-56	M	Extracellular matrix protein
140	A49795	Amyloid beta protein precursor	6e-35	M	Alzheimer's disease
4, 33	AAB51694	Pedal peptide precursor protein	2e-29	S	Neuropeptide
113	NP_001210	Calumenin precursor	1e-54	S	Protein folding and sorting

M; cell membrane, S; secreted.

Table 4. N-terminal amino acid sequence of novel cDNA clones

Clone	Signal peptide sequence	S score	TM
3-4, 11	MITLMMMIIKMMIIIIIMMMIIIMMMMIIIICFW...	0.972	7-29
112	MAVWVLLLVADWSLLLPTAVCQVLPCMMRPQK...	0.952	4-26
45	MHTLNFALSLSLSLSLSLSLS	0.942	-
119	MYSQVVVLLCLFVAACHGFFVPPGYGGHRRPAI...	0.933	-
131	MKPDFGVLSDEEIHYINNIARTSWKAGRNFHPEDHAHVK...	0.926	-
16, 19, 116, 117, 139, 145, 106	MCHPFSSMMSVISVLLLASAVFSAPVEKA...	0.918	-
11-8	MVSTWTVCALLVMELTWSVKAALDCYRGSNVQELQKTC...	0.918	-
42	MLLQTVFLVCLSYAVAGPAYMKYFKIESAMPTYPETKVDKDP...	0.918	-
2, 3-15, 136, 11-15	MLSVSQLLFATAFVVCFVSAHSFCGV...	0.904	-
14, 111	MKEMFLLFVCVLCHWRTGYTLGT...	0.891	2-23
10, 109, 121, 128	MRTAAISVVLLLCAALSQCSGQHSQNEGESQ...	0.867	-
129	MTKSLVLLIVCACIGSSWCSPFTFPLHAS...	0.853	-
3-12	MVVVGWFILLRLYSLLCSLYYRVCFVPSLSRRPA...	0.844	-
143	MGGFFWTTLVCSMSLVIATSARRSYQSSAELE...	0.842	-
3-3	MLLLQCLLFYIVKSCIYEYLIKFYVSYYAP	0.815	1-23
11-14	MIMLIFFTSYYPIITLTIPHTPIYNPFT...	0.762	-
3-11	MAAVQIFCKRMRVGRKRWALLVYVCVCVCVCVCVCVFV...	0.685	18-39
7	MLPSLHISQTQVSLLLKLSMGLLLYCACRLTSAGRP...	0.677	-
3-7	MLCCLSLIFPCFCELLPK-SVSGQLG	0.665	-
134	MFPSHSKFVFSALILQHLATQNSSPR..	0.594	-
3-1	MSGNLFSTVCPILSVILHGTCTKLEPGSSFTTVST...	0.557	-
32	MRFTLEYPFSKTFLTTSFLLMQYIFMHTHTCTHTDYFHS...	0.516	-
8, 36, 108, 132, 120	MWGKKIDDKLCYKINIRVTKGILFLSLSLSLSLSLSL...	0.506	16-38
127	MFLCLLTPGIPDTVGVERANKVVLTDVEW...	0.487	-
1, 103	MQYLSETLSSMSNLSSLIALPLILLNMTNQTSANR...	0.485	-
138	MLLRMDMRYINAIIIIIIINTHRRIPIQIHTHTHTHID...	0.330	1-22
31	MALGKKDCLCRNQTNSYEDKICFLSLSLSLSLSLSLSPP...	0.302	21-37
1-1	MQKKKEVNSSYDKHHFLTDHRLTTKDQDLIQKSSQNMKSVYFWG...	0.279	37-59
3-10	MSTAQLVKLVKTHQSNKPVAPVDNSSMRVAVGSS...	0.132	168-189
35	MYDSSIWMIGECQLEPSPHKIFKASFVNDVTETELSPQRKRR...	0.098	80-102
11-1, 11-9, 122, 15, 105	MMTLGDVYFIISFIFSLLLP	-	1-20

*indicates the numbers of amino acid residues spanning the membrane.

Discussion

In *Aplysia*, many membrane proteins participate in synaptic function. To screen these membrane proteins efficiently we used the yeast-based signal sequence trap method. The validity of this approach was first proven by using pSMASH-5HT2c, which contained the N-terminal of a 5HT receptor molecule.

Our results show that the transmembrane domain can function as a signal sequence. This is consistent with the previous findings which found that the cDNA isolated by the signal sequence trap method encoded a portion of the seven transmembrane serotonin receptor (Klein et al., 1996). For example, clone #3-10 in our study has a long cytoplasmic domain from residue 1 to 167, followed by one transmembrane domain from residue 168 to 189. Invertase was fused on the extracellular loop, thereby allowing survival on sucrose media without a signal sequence.

Eighty three percent of the 103 selected clones, homologous to known proteins or novel proteins, were found to be secreted or membrane proteins. Twenty four percent of these secreted or membrane proteins appeared to possess a transmembrane domain according to the prediction program (PSORT II and SOSUI). The other 17% of the selected clones were false positives, which is a similar percentage to that reported previously by this method (Jacobs et al., 1997; Taft et al., 2002). However, it is possible that some of these 18 clones, with a signal score below 0.48 encode secreted or membrane proteins, since the signal peptide prediction program (SignalP) is only 75-80% accurate (Klein et al., 1996). The present study demonstrates that the yeast-based signal sequence trap method could efficiently select the secreted or membrane proteins of the *Aplysia* central nervous system.

Acknowledgments

This work was supported by grant No. 1999-1-213-002-5 from the Basic Research Program of the Korea Science & Engineering Foundation and by grant No. M1-0104-00-0140 under the NRL Program and M1-0108-00-0075 from MOST. M.-J. Kim was supported by BK21 Fellowship from the Korean Ministry of Education.

References

Carlson M, Taussig R, Kustu S, and Botstein D (1983) The

secreted form of invertase in *Saccharomyces cerevisiae* is synthesized from mRNA encoding a signal sequence. *Mol Cell Biol* 3: 439-447

Goo JH, Ahn Y, Park OK, and Park WJ (1999a) Selection of Drosophila genes encoding secreted and membrane proteins. *Mol Cells* 9: 564-568.

Goo JH, Park AR, Park WJ, and Park OK (1999b) Selection of *Arabidopsis* genes encoding secreted and plasma membrane proteins. *Plant Mol Biol* 41: 415-423.

Hamada T, Tashiro K, Tada H, Inazawa J, Shirozu M, Shibahara K, Nakamura T, Martina N, Nakano T, and Honjo T (1996) Isolation and characterization of a novel secretory protein, stromal cell-derived factor-2 (SDF-2) using the signal sequence trap method. *Gene* 176: 211-214.

Jacobs KA, Collins-Racie LA, Colbert M, Duckett M, Golden-Fleet M, Kelleher K, Kriz R, La Vallie ER, Merberg D, Spaulding V, Stover J, Williamson MJ, and McCoy JM (1997) A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* 198: 289-296

Kaiser CA, and Botstein D (1986) Secretion-defective mutations in the signal sequence for *Saccharomyces cerevisiae* invertase. *Mol Cell Biol* 6: 2382-2391.

Klein RD, Gu O, Goddard A, and Rosenthal A (1996) Selection for genes encoding secreted proteins and receptors. *Proc Natl Acad Sci USA* 93: 7108-7113

Kristoffersen P, Teichmann T, Stracke R, and Palme K (1996) Signal sequence trap to clone cDNAs encoding secreted or membrane-associated plant proteins. *Anal Biochem* 243: 127-132

Nakamura T, Tashiro K, Nazarea M, Nakano T, Sasayama S, and Honjo T (1995) The murine lymphotoxin-β receptor cDNA: Isolation by the signal sequence trap and chromosomal mapping. *Genomics* 30: 312-319

Taft RA, Denegre JM, Pendola FL, and Eppig JJ (2002) Identification of genes encoding mouse oocyte secretory and transmembrane proteins by a signal sequence trap. *Biol Reprod* 67: 953-960

Tashiro K, Tada H, Heilker R, Shirozu M, Nakano T, and Honjo T (1993) Signal sequence trap: A cloning strategy for secreted proteins and type I membrane proteins. *Science* 261: 600-603.

von Heijne G (1985) Signal sequences. The limits of variation. *J Mol Biol* 184: 99-105.

[Received May 21, 2003; accepted April 3, 2003]