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Neuroscience Letters 343 (2003) 134–138

Neuroscience Letters

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Identification of nuclear/nucleolar localization signal in *Aplysia* learning associated protein of slug with a molecular mass of 18 kDa homologous protein

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Received 25 January 2003; received in revised form 14 February 2003; accepted 21 February 2003

Abstract

We isolated a learning associated protein of slug with a molecular mass of 18 kDa (LAPS18) homologue from the expressed sequence tag database of *Aplysia kurodai* and named it *Aplysia* LAPS18-like protein (ApLLP). ApLLP encodes 120 amino acids and has 57% identity with LAPS18. To examine the subcellular expression pattern of ApLLP we constructed an EGFP-tagged ApLLP fusion protein and overexpressed it in both *Aplysia* neurons and COS-7 cells. In contrast to the previous findings, which showed that LAPS18 is secreted by COS-7 cells, ApLLP-EGFP was localized to the nucleus, and most of it to nucleoli. Analysis of deletion mutants of ApLLP-EGFP showed that the N-terminal and the C-terminal nucleolar and nucleus localization signal sequences are important for localization to the nucleus and the nucleoli.

Keywords: LAPS18; Nucleus; Nucleolus; Nucleolar localization signal sequence; Nuclear localization signal sequence

Long-term memory formation requires new mRNA and protein synthesis. Many studies have attempted to elucidate the molecular mechanism underlying long-term memory using the simpler invertebrate nervous system and the mammalian hippocampus [1,12,15]. Recently, Nakaya and colleagues isolated 'Learning Associative Protein of Slug' with a molecular mass of 18 kDa (LAPS18) from *Limax marginatus* [13]. They showed that the expression of LAPS18 was induced by the odor–taste associative learning, which evoked an odor avoidance by the association of carrot juice smell (conditioned stimulus) and quinidine sulphate solution (unconditioned stimulus), and that this protein may be involved in cell migration and adhesion. However, the physiological function and the detailed subcellular localization of LAPS18 have not been thoroughly investigated.

The subcellular localizations of proteins can be regulated by interactions between diverse signal sequences and their receptor proteins. Nuclear targeting is also mediated by nuclear localization signal sequences (NLSs) and by cognate receptors of the importin/karyopherin superfamily [14]. The NLSs can be classified into two groups: monopartite and bipartite. Monopartite classical NLSs consist of a short stretch of basic lysine/arginine-rich residues, NLS of the SV40 large T-antigen being the best-known example [7]. Bipartite NLSs are composed of two strings of basic amino acids separated by a 10–12 amino acid intervening sequence [2].

In the present study, we first isolated the full-length cDNA of *Aplysia* LAPS-like protein from an *Aplysia kurodai* expressed sequence tag (EST) database [10]. Sequence analysis showed that *Aplysia* LAPS18-like protein (ApLLP) has an open reading frame of 360 bp encoding a deduced polypeptide of 120 amino acids and a 57% identity with the LAPS18 amino acid sequence cloned from *L. marginatus* (Fig. 1A,B). By using the PSORT WWW server (http://psort.ims.u-tokyo.ac.jp) we found that ApLLP has a lysine-rich domain in the C-terminal region and two putative NLSs; one of these is similar to monopartite NLS (KHRR, amino acids 8–11) in its N-terminal and the other is a bipartite NLS (**KK**QKVKVAKLKT**KKKIG**, amino acids 109–115) in the C-terminal region. The N-terminal sequence of ApLLP

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doi:10.1016/S0304-3940(03)00269-6

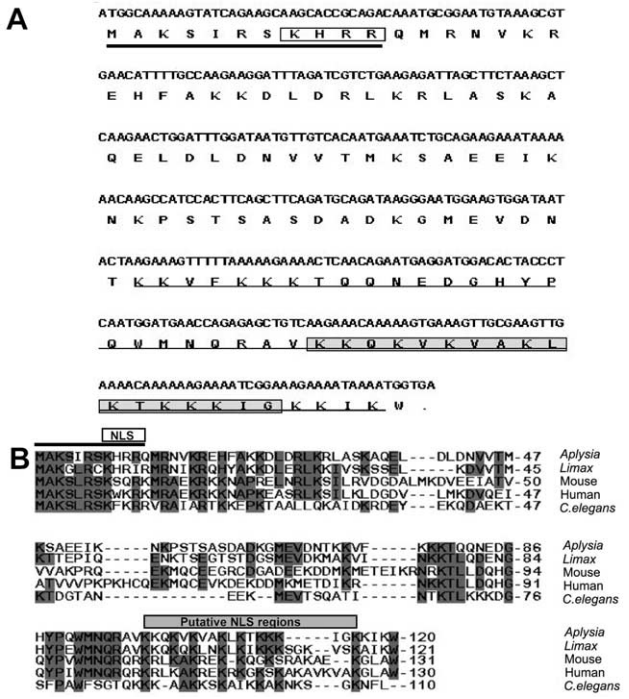

Fig. 1. cDNA and the deduced amino acid sequences of ApLLP. (A) The open and shaded boxes indicate the NLSs, which are predicted to Pat4 and bipartite, respectively. A thick underline indicates the signal peptide sequence. The lysine-rich domain is indicated by a thin underline. (B) Amino acid alignment of ApLLP, *Limax* LAPS18 (GenBank accession number AAG35713), and human (AAH06002), mouse (XP_193311) and *Caenorhabditis elegans* (AAF99893) homologues. The dark shaded box indicates the position of the conserved amino acids. Signal peptide sequences are indicated by a thick line at the N-terminus.

is similar, but not identical to that of LAPS18 which was claimed to be a signal peptide sequence for secretion [13] (Fig. 1A).

A search of the GenBank database using BLAST revealed several homologous proteins in the human (GenBank accession number AAH06002), mouse (XP_193311), and nematode (AAF99893). A multiple alignment of the homologous proteins showed that other homologues also have the putative signal peptide sequence and NLSs (Fig. 1B). However, the N-terminal NLS is absent from the *Limax* and nematode *Caenorhabditis elegans* homologues, which suggests that each homologue could localize to different subcellular compartments.

To study the role of these NLSs and the subcellular localization of ApLLP in the *Aplysia* neuron, ApLLP was fused to the N-terminus of EGFP (Fig. 2A) and then subcloned into the neuronal expression vector pNEXδ [6]. The resulting construct was injected into *Aplysia* pedal neurons and incubated for 48 h at 18 °C. Fluorescence was determined by confocal microscopy. Since LAPS18 is reported to be a secretory protein [13], we initially expected to detect ApLLP fluorescence in the *trans*-Golgi network of the cytoplasm or in the plasma membrane, because typical secretory proteins are delivered through these structures. Surprisingly, no ApLLP-EGFP was detected in the *trans*-Golgi network or in the cytoplasm (Fig. 2Bb). Instead, ApLLP-EGFP was found to be localized to the nucleus, particularly in nuclear bodies of cultured *Aplysia* neuron (Fig. 2Bb). The ApLLP-EGFP expression was detected as early as 2 h 30 min in a similar subcellular localization pattern and was maintained at least up to 48 h after DNA

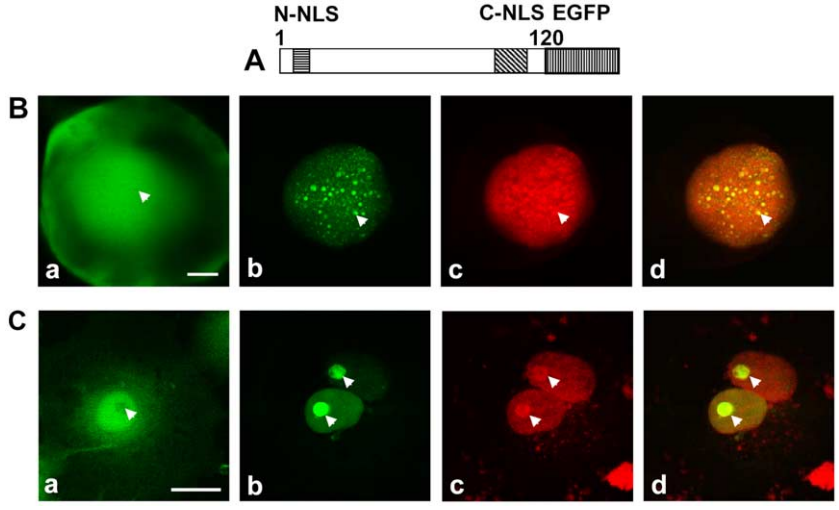

Fig. 2. Subcellular localization of ApLLP-EGFP. (A) A schematic view of the structure of ApLLP-EGFP. Putative NLSs are indicated by boxes. ApLLP was amplified by polymerase chain reaction (PCR) using specific primers 5′-CGAAGCTTGCCACCACCATGGCAAAAAGTATCAGA-3′ and 5′-CCTCTAGACCATTTTATTTTCTTTCC-3′ and then subcloned into the *Hin*dIII/*Xba*I sites of the pNEXδ-EGFP to create pNEXδ-ApLLP-EGFP [6]. The cell culture method used and the detection of GFP through the confocal microscope were performed as described previously [9,11]. (B) Subcellular localization of EGFP-tagged ApLLP in *Aplysia* neurons. (a) EGFP alone; (b) ApLLP-EGFP; (c) propidium iodide staining of ApLLP-EGFP expressing neuron; (d) merged image of (b) and (c). (C) Subcellular localization of EGFP-tagged ApLLP in a transfected COS-7 cell. COS-7 cells were transfected with 1 μg of deletion or fusion constructs using Lipofectamine (Life Technologies) in serum-free DMEM for 3 h. The medium was changed and incubated for 24–36 h after each transfection. (a) EGFP alone; (b) ApLLP-EGFP; (c) propidium iodide staining of an ApLLP-EGFP expressing neuron; (d) merged image of (b) and (c). Arrowheads indicate representative nucleoli. Scale bar, 10 μm.

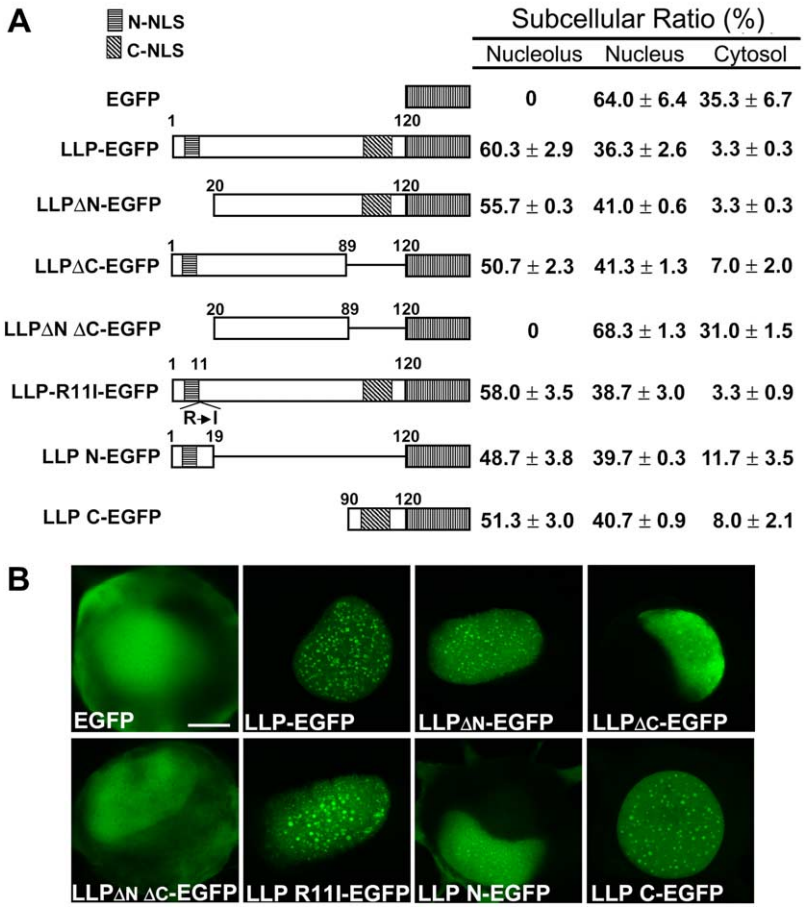

Fig. 3. Characterization of the NLS of ApLLP. (A) Diagrams of EGFP-tagged ApLLP deletion mutants and their relative subcellular localization ratios. DNAs for various mutant ApLLP fragments were prepared by PCR using ApLLP cDNA as template. The nucleotide sequences of primers used for PCR are as follows: primer 1, 5′-CGAAGCTTGCCACCACCATGGCAAAAAGTATCAGA-3′; primer 2, 5′-CCTCTAGACCATTTTATTTTCTTTCC-3′; primer 3, 5′-CGAAGCTTGCCACCACCATGCATTTTGCCAAGAAGGA-3′; primer 4, 5′-CGTCTAGAGTAGTGTCCATCCTCAT-3′; primer 5, 5′-CGTCTAGATTCACGCTTTACATTCC-3′; primer 6, 5′-AGAAGCTTGCCACCATGCCTCAATGGATGAACCAG-3′; primer 7, 5′-AGAAGCTTGCCACCATGGCAAAAAGTATCAGAAGCAAGCACCGCATCCAAATG-3′; primers 2 and 3 for the DNA fragment LLPΔN which corresponds to 20–120 amino acids of ApLLP; primers 1 and 4 for the DNA fragment LLPΔC which corresponds to 1–89 amino acids of ApLLP; primers 3 and 4 for the DNA fragment LLPΔNΔC which corresponds to 20–89 amino acids of ApLLP; primers 1 and 5 for the DNA fragment LLP N which corresponds to 1–19 amino acids of ApLLP; primers 2 and 6 for the DNA fragment LLP C which corresponds to 90–120 amino acids of ApLLP; and primers 2 and 7 for the DNA fragment LLP R11I which corresponds to the point mutated full-length amino acids of ApLLP. To construct each DNA fragment fused with EGFP, the amplified DNA fragments were digested with *Hin*dIII and *Xba*I (underlined in the primer sequences) and then subcloned into the *Hin*dIII and *Xba*I site of pNEXδ-EGFP. The fluorescence intensities of the subcellular compartments were obtained using the 'line-profile' method in this software, and subcellular ratios (%) of GFP fluorescence were calculated by dividing the fluorescence of each subcellular region by the total GFP fluorescence of the cell. Data represent the mean ± SEM; $n = 3$ cells. (B) Subcellular localization of EGFP-tagged ApLLP mutants in *Aplysia* neurons. Scale bar, 15 μm.

microinjection. To identify these nuclear bodies, we stained cells using propidium iodide, which is known to stain the nucleus and the nucleolus [4,5]. EGFP fluorescence co-localized with propidium iodide in ApLLP-EGFP expressing neurons (Fig. 2Bb–d). In addition, the size of these nuclear bodies containing ApLLP-EGFP was similar to that of *Aplysia* nucleoli (~1.8 μm), as determined by electron microscopy (data not shown), and it has been previously reported that *Aplysia* neurons contain numerous nucleoli [8]. These findings suggest that the nuclear bodies, which were co-localized with ApLLP-EGFP, are nucleoli. In the EGFP control, fluorescence was detected diffusely over the entire cell body, but no fluorescence was detected in nucleoli (Fig. 2Ba). These results suggest that ApLLP has a functional nucleus and nucleolus localization signal.

In order to verify ApLLP's nuclear/nucleolar localization, we transfected pNEXδ-ApLLP-EGFP into COS-7 cells, which have a larger nucleus and prominent nucleoli. In the transfected COS-7 cell, ApLLP-EGFP was also localized in the nucleus and the nucleoli as in the *Aplysia* pedal neuron (Fig. 2Cb–d). In EGFP expressing COS-7 cells, fluorescence was diffuse throughout the cell except for the nucleolus (Fig. 2Ca). These results show that ApLLP localizes to the nucleus and mainly to the nucleoli.

To map the minimal determinants required for nuclear and nucleolar targeting, we constructed a series of

expression plasmids that contained various ApLLP deletion mutants fused to EGFP (Fig. 3A). LLPΔN-EGFP and LLPΔC-EGFP still localized to the nucleus and nucleoli, though the C-terminal deletion mutant showed weaker expression to the nucleoli and stronger expression in the cytoplasm (Fig. 3A,B). In contrast, LLPΔNΔC-EGFP, lacking both the N- and C-terminal NLSs, was found to be distributed throughout the cytoplasm and in the nucleus but no expression was detected in nucleoli, a distribution pattern resembling that of EGFP (Fig. 3A,B). These results suggest that the regions from amino acids 1 to 19 and from 90 to 120 contain functional nucleus and nucleolus localizing signals.

Nakaya and coworkers have shown that *Limax* LAPS18 is a secretory protein which has a putative signal peptide sequence in its N-terminal region, and amino acid sequence analysis shows that *Limax* LAPS18 contains only one putative NLS in its C-terminal region. In addition to the C-terminal NLS, ApLLP was found to have an additional putative NLS instead of the signal peptide sequence in its N-terminal region (Fig. 1B). Thus, it is possible that the presence of this additional N-terminal NLS could direct ApLLP to the nucleus/nucleolus. To examine this possibility, we mutated the N-terminal NLS of ApLLP to resemble a putative signal peptide sequence of *Limax* LAPS18 by replacing the 11th amino acid residue of ApLLP arginine by isoleucine of LAPS18 (LLP R11I-EGFP). As shown in Fig. 3A,B, this mutation in the N-terminal sequence of ApLLP still did not affect the normal targeting to the nucleus and nucleolus, indicating that the mutation R11I did not produce a functional signal peptide sequence for the secretory pathway.

To examine whether the putative NLS and nucleolar localization signal sequence regions are sufficient to direct a reporter protein to the nucleus/nucleolus, the N-terminal or the C-terminal region of ApLLP was fused to EGFP and subcloned into pNEXδ (LLP N-EGFP, LLP C-EGFP). Both constructs displayed nucleus and nucleolus localization in *Aplysia* pedal neurons. However, the fluorescent intensities of the N-terminal and C-terminal fusion constructs in subcellular compartments differed (Fig. 3A,B). LLP N-EGFP expressing neurons showed less fluorescence in the nucleolus and more fluorescence in the cytoplasm than full-length ApLLP expressing neurons. Nevertheless, LLP C-EGFP had a distribution pattern similar to that of full-length ApLLP (Fig. 3A,B).

Similar localization patterns were observed in transfected COS-7 cells, but both N-terminal and C-terminal NLS-deleted ApLLP-EGFP showed a diffusely distributed localization pattern observed for EGFP expressing COS-7 cells. LLP N-EGFP and LLP C-EGFP localized in the nucleus and in the nucleolus in COS-7 cells (data not shown). Moreover, an inspection of the expression patterns of the ApLLP-EGFP variants revealed that both the N-terminal and the C-terminal region are individually capable of providing adequate nuclear and nucleolus localization signals, but that the C-terminal provides the stronger signals.

The nucleolus is a subcellular organelle containing the rRNA genes and ribosome biogenesis factors. Considering the role of the nucleolus, it is possible that ApLLP is involved in rRNA transcription and ribosome assembly. Recent studies suggest that the nucleolus has very diverse functions, for example, protein retention, RNA transport, RNA modification and cell cycle modification in addition to rRNA transcription and ribosome assembly [3]. Thus, it is tempting to suggest that ApLLP might be involved in the protein synthesis required for memory formation, since LAPS18 was isolated as a learning associated gene.

Acknowledgements

This work was supported by grants M1-0104-00-0140 under the National Research Laboratory Program and M1-0108-00-0075 from the Ministry of Science and Technology, and No. R01-1999-000-00147-0 from the Korea Science and Engineering Foundation. H.K., D.-J.C. and Y.-S.L. were supported by a Brain Korea 21 Research Fellowship.

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