Regulation of the Level of Vesl-1S/Homer-1a Proteins by Ubiquitin-Proteasome Proteolytic Systems*

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The vesl-1S/homer-1a gene is up-regulated during seizure and long term potentiation. Other members of the Vesl family, Vesl-1L, -2, and -3, are constitutively expressed in the brain. We examined the regulatory mechanisms governing the expression level of Vesl-1S protein, either an exogenously introduced one in COS7 or human embryonic kidney 293T cells or an endogenous one in rat brain neurons in cultures. In both cases, application of proteasome inhibitors increased the amount of Vesl-1S protein but not that of Vesl-1L, -2, or -3 protein. Deletion analyses revealed that the C-terminal 11-amino acid region was responsible for the proteolysis of Vesl-1S by proteasomes. Application of proteasome inhibitors promoted ubiquitination of Vesl-1S protein but not that of the Vesl-1S deletion mutant, which evaded proteasome-mediated degradation. These results indicate that ubiquitin-proteasome systems are involved in the regulation of the expression level of Vesl-1S protein.

Long term potentiation (LTP),¹ which is thought to underlie mechanisms of learning and memory, has two distinct phases. The early-phase LTP lasts for no more than several hours and does not depend on protein synthesis, whereas the late-phase LTP (L-LTP) lasts for weeks in vivo and depends on de novo RNA transcription and protein synthesis (1–3). The formation of long term memory requires de novo RNA transcription and protein synthesis (4–6). Thus, activity-dependent gene expression is expected to play a critical role in long term memory.

Vesl-1S/Homer-1a was isolated as a gene whose expression was up-regulated following LTP induction (7, 8). Vesl-1L/Homer-1c/PSD-Zip45 and Vesl-1L/Δ12/Homer-1b, which are splice variants of Vesl-1L, are constitutively expressed in the brain. Vesl-2/Homer-2b, Vesl-2/Δ11/Homer-2a, and Vesl-3/Homer-3 are highly related to Vesl-1L in that both contain EVH1 domains in their N termini and leucine zippers in their C termini that mediate multimerization (9–11). The EVH1 domains of Vesl family proteins interact with group I metabotropic glutamate receptors 1/5 (7) and inositol triphosphate receptors (12). Moreover, Vesl family proteins interact with the Shank protein, which binds to the NMDAR-PSD-95-GKAP complex and cortactin (13, 14). Thus Vesl family proteins may be a component of huge PSD-95 protein complexes located in postsynaptic regions.

The level of vesl-1S mRNA in the hippocampus is drastically increased during seizure and LTP, but the increase in the amount of Vesl-1S protein is limited (9). Moreover, all members of Vesl family contain PEST sequences that are thought to be ubiquitin-proteasome-dependent degradation signals (15). We considered that the amount of Vesl-1S protein might be regulated by certain proteases. The ubiquitin-proteasome pathway, one of the protein degradation systems of the cell, is involved in a variety of cellular processes, for instance, cell cycling (16), transcriptional activation (17), apoptosis (18), circadian rhythm (19), neurodegeneration (20), and neuronal plasticity (21, 22). Protein ubiquitination involves three classes of enzymes, E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes, and E3 ubiquitin-protein ligases. In these multienzyme pathways, target proteins are conjugated with polymers of ubiquitin, which trigger their rapid degradation by proteasomes (23). As the expression level of vesl-1S mRNA does not readily parallel that of Vesl-1S protein after L-LTP induction, we investigated the effects of protease inhibitors on the amount of Vesl-1S protein. We found that proteasome inhibitors promoted the expression and ubiquitination of Vesl-1S proteins and identified a proteolytic signal sequence that controlled its ubiquitinations.

EXPERIMENTAL PROCEDURES

Chemicals—E-64-d (2S,3S-t-epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester) and MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucine) were purchased from Peptide Institute Inc. (Osaka, Japan). Lac-tactin was purchased from Calbiochem. These compounds were dissolved in Me2SO before use, and throughout the experiments, the final concentration of Me2SO in cell culture medium, including control culture medium, was kept at 0.1%.

Cell Culture—For Western blot analyses, cortical cells were used. Rat neurons were cultured as follows. Brains of embryonic Wistar rats (E18–19) were rapidly removed, dissected, and incubated at 37°C for 10 min in papain solution containing the following: 5 mM L-cysteine, 1 mM EDTA, 10 mM HEPES-NaOH (pH 7.3), 100 μg/ml bovine serum albumin, 10 units/ml papain (Worthington), and 0.02% DNase (Sigma). The reaction was stopped by adding an equal volume of heat-inactivated horse serum (Life Technologies, Inc., Grand Island, NY). Cells were filtered through lens paper, plated on polyethylenimine-coated 60-mm dishes at 8 × 10⁶–4 × 10⁶ cells/dish, and cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units of penicillin G/ml, 10 μg of streptomycin sulfate/ml, 4 mM glutamine, and 10% horse serum.

The COS7 cells and the HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 100 units of penicillin G/ml, 10 μg of streptomycin sulfate/ml, 4 mM glutamine, and 10% (v/v) fetal bovine serum.

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¹ The abbreviations used are: LTP, long term potentiation; L-LTP, late-phase LTP; HEK, human embryonic kidney; HA, hemagglutinin; NPT, neomycin phosphotransferase; GST, glutathione S-transferase.
RESULTS

The Amount of Vesl-1S Protein, but Not That of Vesl-1L, -2, or -3, Is Increased by Proteasome Inhibitors—To investigate the turnover of proteins of the Vesl family, we constructed plasmids that expressed Vesl proteins containing FLAG tags (Fig. 1A). These constructs did not contain untranslated regions. The expression of these mRNAs was regulated by the same promoter, and these Vesl proteins were translated by the same initiation signal. The plasmids were introduced into COS7 cells. The effects of protease inhibitors on the levels of Vesl proteins were evaluated by immunoblotting using anti-FLAG antibody. We used specific inhibitors for proteasomes and lysosomal proteases. Two types of proteasome inhibitors, MG132 and lactacystin, which are structurally unrelated, significantly increased the amount of Vesl-1S protein, whereas the amounts of Vesl-1L, -2, and -3 were not affected. An inhibitor of proteases of the lysosomes and calpain family, E-64-d, had no effect on the amount of any protein of the Vesl family (Fig. 1, B and C). These results indicate that Vesl-1S, but not Vesl-1L, -2, or -3, undergoes rapid degradation by proteasomes.

Fig. 1. Vesl-1S, but not Vesl-1L, -2, or -3, is degraded by proteasomes. A, schematic structures of members of the Vesl family of proteins. Closed boxes indicate FLAG tags. Hatched boxes indicate PEST sequences. Vesl-1S and Vesl-1L have two PEST sequences (PEST scores, 11.53 and 5.64, respectively). Vesl-2 and -3 have one PEST sequence each (PEST scores, 14.23 and 5.84, respectively). B, Western blot analyses of Vesl proteins from COS7 cells. COS7 cells were transfected with FLAG-tagged vesl family cDNA, and these cells were cultured with 0.1% fetal bovine serum. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO2.

Construction of FLAG-tagged Proteins and HA-tagged Ubiquitin—FLAG (DYKDDDK)-tagged Vesl constructs and Vesl-1S deletion mutants were generated by polymerase chain reaction using specific primers and cloned into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA), which contains the neomycin resistance gene (NPT II). FLAG tags were inserted between the initiation codon and the codon for the second amino acid to construct FLAG-Vesl-1L, -1S, -2, and -3. FLAG-tagged Lc had been prepared as described (24).

N-terminal HA-tagged ubiquitin was generated by the polymerase chain reaction using high fidelity thermostable DNA polymerase from Roche (Tokyo, Japan) with the following primers: 5'-ATAGATCGCTCGAGCCATGGCATACCCATTACGAGCCACCTACGATCT-TCGTGAAACTCCCTAC-3' and 5'-ATAGATCTTTTACACCCACCTCGAGCACCAGAGC-3'. The polymerase chain reaction product was digested with EcoRI, subcloned into pBluescript II SK+, and sequenced. pBluescript II SK+ was digested with EcoRI and XhoI and subcloned into pcDNA3.

COS7 Cell Transfections and Immunoblots—Transfections of DNA constructs into COS7 cells were performed with 50 μg of each plasmid DNA by electroporation (Electro Cell Manipulator 600; BTX) according to the manufacturer's instructions. 48 h after transfection, cells were treated with drugs for 10 h and then cells were extracted in 2× SDS sample buffer. Equal amounts of cell extract were separated by SDS polyacrylamide gel electrophoresis (12.5% polyacrylamide) after transfer of the separated proteins to a polyvinylidene difluoride membrane, the membrane was fixed for 45 min with 4% paraformaldehyde in phosphate-buffered saline at 4 °C and rinsed three times for 20 min with phosphate-buffered saline. FLAG-tagged Vesl proteins from transfected COS7 cells were identified by using a monoclonal antibody (anti-FLAG M5 antibody; Eastman Kodak Co.) and the Vistra ECF Western blotting system (Amersham Pharmacia Biotech). Vesl-1S proteins from transfected COS7 cells were detected by using a polyclonal antibody (1B3; MBL, Nagoya, Japan). Vesl proteins were evaluated by immunoblotting using anti-FLAG antibody. We used specific inhibitors for proteasomes and lysosomal proteases. As the 11-amino acid region is unique to Vesl-1S family of proteins, this sequence most likely predetermines the stability of the Vesl-1S protein. The C-terminal 11-Amino Acid Region of Vesl-1S Is Responsible for Its Degradation by Proteasomes—The next step in our study was to identify the region of the Vesl-1S protein responsible for the regulation of its degradation by proteasomes. We introduced deletion constructs of FLAG-tagged Vesl-1S cDNA into COS7 cells, and the levels of the three truncated Vesl-1S proteins, VSD-1, -2, and -3 (Figs. 2A), were evaluated by immunoblotting using anti-FLAG antibody. The unevenness in the transfection efficiencies of vectors carrying VSD-1, -2, and -3 was normalized to the level of the NPT II protein expressed from the same vector. As Fig. 2, A and C shows, the expression levels of VSD-1, -2, and -3 were remarkably higher than that of Vesl-1S protein. These results indicate that the 11-amino acid region in the C terminus of Vesl-1S reduces the stability of the Vesl-1S protein.

To investigate the mechanism responsible for the increase in the amounts of VSD-1 and -3, we examined the effects of the protease inhibitors MG132, lactacystin, and E-64-d on the levels of these truncated proteins (Fig. 3, A and B). No effect was observed, indicating that the increase in the level of truncated Vesl-1S protein was because of its resistance to proteolysis by proteasomes. As the 11-amino acid region is unique to Vesl-1S among Vesl family proteins, this sequence most likely predetermines Vesl-1S for rapid degradation.

The Amount of Endogenous Vesl-1S Protein Is Increased by Application of a Proteasome Inhibitor to Cultured Neurons—We investigated whether the turnover of endogenous Vesl-1S protein was also affected by the application of proteasome inhibitors to cultured neurons. The amount of Vesl-1S protein was measured by Western blot analysis using the antipeptide Vesl antibody, which recognizes all members of the Vesl family of proteins. As the molecular mass of Vesl-1S protein is significantly lower than that of other members of the Vesl family, we could identify Vesl-1S immunosignals after SDS polyacrylamide gel electrophoresis. The mobility of endogenous vesl proteins was increased by a proteasome inhibitor.
Vesl-1S protein during SDS polyacrylamide gel electrophoresis was confirmed by loading bacterially expressed Vesl-1S side by side on the polyacrylamide gel. After the application of MG132, we observed strong immunosignals at 28 kDa, which is a slightly lower molecular mass than that of the recombinant proteins. No immunosignals at the corresponding position were detected under control conditions. These Vesl-1S immunoreactivities were blocked by incubation of the anti-Vesl antibody with the GST-Vesl-1S protein (Fig. 4). MG132 did not affect the intensity of immunosignals at 48 kDa, which corresponded to those of Vesl-1L, -2, and -3. These results indicate that only endogenous Vesl-1S among Vesl family members is selectively degraded by proteasomes in cultured neurons and strongly suggest that the level of Vesl-1S protein is regulated by the proteasome pathway in neurons.

**Ubiquitination of Vesl-1S Protein**—We next investigated whether the degradation of Vesl-1S protein was regulated by ubiquitin signals. To investigate its ubiquitination, we co-expressed FLAG-tagged Vesl-1S or Vesl-1S mutants with HA-tagged ubiquitin in HEK293T cells and examined their interaction by immunoprecipitation with anti-FLAG antibody. We found that the treatment of cells with the proteasome inhibitor (MG132) not only led to the accumulation of Vesl-1S protein but also promoted the accumulation of multiubiquitinated Vesl-1S. In contrast, the deletion mutant VSD-3, which was not degraded by proteasomes, was not altered to multiubiquitinated forms by the treatment of proteasome inhibitor (Fig. 5A). It is known that ubiquitin is attached to the lysine residues in target proteins (25). The C-terminal Vesl-1S-specific region, which is responsible for proteolysis of Vesl-1S by proteasomes, contains one lysine residue. To examine whether this lysine residue is essential for ubiquitination of Vesl-1S, we constructed a Vesl-1S mutant in which this lysine residue was replaced with an arginine residue (V1S-K186R; see Fig. 5B). We found that the ubiquitination and the expression of the V1S-K186R were both promoted by the proteasome inhibitor. There was little difference in the extent of ubiquitination be-
nated V1S-K186R is shown relative to that of Vesl-1S. The biquitinated V1S-K186R normalized by the intensity of non-ubiquitinated Vesl-1S and V1S-K186R depicted in the Vesl-1S-specific region. A, immunoprecipitation analyses of Vesl-1S proteins from HEK293T cells. HEK293T cells were transfected with FLAG-tagged Vesl-1S, VSD-3, or IeBa (positive control), in combination with HA-tagged ubiquitin, and these cells were cultured with 0.1% Me2SO or 10 μM MG132 for 10 h. Cell lysates were subjected to immunoprecipitation (IP) with FLAG antibody, and the resulting precipitates were subjected to immunoblot (IB) analyses with antibodies to ubiquitin or FLAG. The high molecular mass ubiquitinated protein, V1S-K186R, with a Lys to Arg mutation in the C-terminal 11-amino acid region, was heavily ubiquitinated. In contrast, the proteasome-resistant stable mutant of Vesl-1S, VSD-3, was not ubiquitinated. Therefore the ubiquitination signals may most likely reside in the C-terminal 11-amino acid region, and the ubiquitination of Vesl-1S may promote the rapid degradation of the protein by proteasomes. At present, we have not identified the sites of ubiquitination, but at least the C-terminal 11-amino acid region does not seem to contain the only or major ubiquitination site. Thus, this region may function as a signal to stimulate ubiquitination of the other sites of Vesl-1S protein.

The level of endogenous Vesl-1S protein in neurons is also regulated, at least partly, by proteasomes. The level of vesl-1S mRNA is increased after L-LTP induction, although the expression of vesl-1L mRNA is not modulated during L-LTP. After L-LTP induction, the level of vesl-1S mRNA is higher than that of vesl-1L mRNA, although the amount of Vesl-1S protein is lower than that of Vesl-1L protein (9). Following L-LTP induction, Vesl-1S proteins accumulated in the portion of the dendrites that had undergone synaptic activation (26). The mRNA for Arc, which was isolated as a synaptic plasticity-regulated gene, is localized to the active postsynaptic regions of dendrites (27) (28). In contrast, vesl-1S mRNA remains in the cell body after L-LTP induction (8). It is likely that newly synthesized Vesl-1S proteins are rapidly degraded by proteasomes following L-LTP induction and that the overall amount of Vesl-1S protein is relatively low. However, when Vesl-1S proteins evade proteasome-mediated degradation by some unknown mechanism, these proteins may accumulate in postsynaptic regions.

An unresolved issue is how proteasome-mediated degradation of Vesl-1S proteins is prevented. What is the inhibition signal? The stability of proteins degraded by proteasomes is regulated by phosphorylation in many cases. Recently, we found that phorbol esters (phorbol 12-myristate 13-acetate or phorbol 12,13-dibutyrate) promoted the punctate distribution of Vesl-1S in neurons and that these phenomena were observed in the absence of de novo protein synthesis.² Phorbol esters activate several types of proteins (29). As some proteins (Mos and p53) evade proteasome-mediated degradation when phosphorylated (30–33), it is possible that the application of phorbol esters may provoke the same phenomena as those induced by proteasome inhibitors in neurons. The Vesl-1S protein may be modified by some kind of kinases activated by phorbol esters and, thereby, evade degradation by proteasomes. A possible hypothesis is that Vesl-1S protein accumulates selectively in certain synapses when proteasomes are unable to degrade the modified Vesl-1S protein present at these synapses. The accumulation of Vesl-1S protein at such synapses might affect the

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cell surface expression of group I metabotropic glutamate receptors 1/5 and promote remodeling of synapses, considering the recent observations that the cell surface expression of group I metabotropic glutamate receptors 1/5 were increased when co-expressed with Vesl-1S and that this increase was inhibited by Vesl-1L (34, 35).

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