Interaction of NE-dlg/SAP102, a Neuronal and Endocrine Tissue-specific Membrane-associated Guanylate Kinase Protein, with Calmodulin and PSD-95/SAP90

A POSSIBLE REGULATORY ROLE IN MOLECULAR CLUSTERING AT SYNAPTIC SITES*

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Norio Masuko‡‡, Keishi Makino‡‡, Hiroaki Kuwahara‡‡, Kohji Fukunaga‡‡, Tamotsu Sudo‡‡, Norie Araki‡‡, Hideyuki Yamamoto‡, Yuji Yamada‡, Eishichi Miyamoto‡, and Hideyuki Saya‡‡

From the Departments of |Tumor Genetics and Biology and ¥Pharmacology, Kumamoto University School of Medicine, 2-2-1, Honjo, Kumamoto 860-0811, Japan and the ¥Cancer Research Laboratory, Hanno Research Center, Taiho Pharmaceutical Co., Ltd., 1-27, Misugidai, Hanno, Saitama 357-8527, Japan

NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase family protein, is known to bind to C-terminal ends of N-methyl-D-aspartate receptor 2B (NR2B) through its PDZ (PSD-95/Dlg/ZO-1) domains. NE-dlg/SAP102 and NR2B colocalize at synaptic sites in cultured rat hippocampal neurons, and their expressions increase in parallel with the onset of synaptogenesis. We have identified that NE-dlg/SAP102 interacts with calmodulin in a Ca\(^{2+}\)-dependent manner. The binding site for calmodulin has been determined to lie at the putative basic α-helix region located around the src homology 3 (SH3) domain of NE-dlg/SAP102. Using a surface plasmon resonance measurement system, we detected specific binding of recombinant NE-dlg/SAP102 to the immobilized calmodulin with a K\(_d\) value of 44 nM. However, the binding of Ca\(^{2+}\)/calmodulin to NE-dlg/SAP102 did not modulate the interaction between PDZ domains of NE-dlg/SAP102 and the C-terminal end of rat NR2B. We have also identified that the region near the calmodulin binding site of NE-dlg/SAP102 interacts with the GUK-like domain of PSD-95/SAP90 by two-hybrid screening. Pull down assay revealed that NE-dlg/SAP102 can interact with PSD-95/SAP90 in the presence of both Ca\(^{2+}\) and calmodulin. These findings suggest that the Ca\(^{2+}\)/calmodulin modulates interaction of neuronal membrane-associated guanylate kinase proteins and regulates clustering of neurotransmitter receptors at central synapses.

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‡ To whom correspondence should be addressed. Tel.: 81-96-373-5116; Fax: 81-96-373-5120; E-mail: hsaya@gpo.kumamoto-u.ac.jp.

The abbreviations used are: PSD, postsynaptic density; DTT, dithiothreitol; NMDA, N-methyl-D-aspartate; NR2B, NMDA receptor 2B; dlg, Drosophila disc large; GST, glutathione S-transferase; RU, resonance unit; SH3, src homology 3; GUK, guanylate kinase; MAGUK, membrane-associated GUK; APC, adenomatous polyposis coli.

protein kinase II (4). Isolated PSDs have also been shown to be enriched in N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors. Recently, several proteins, including PSD-95/SAP90 (5), NE-dlg/SAP102 (6, 7), and chapsyn-110/PSD-93 (8), which belong to a novel protein family called membrane-associated guanylate kinases (MAGUKs), have been identified as components of PSD. These MAGUK proteins are homologous to the Drosophila disc large (dlg) tumor suppressor protein (6, 7) in both sequence and structural organization and contain three distinct domains: an N-terminal segment comprising one or three copies of ~80–90 amino acid motif called PDZ (PSD-95/Dlg/ZO-1) domain, a src homology 3 (SH3) domain, and a region with high similarity to guanylate kinases (9, 10). These domains have been shown to be utilized as modules for interacting with various cellular proteins.

PSD-95/SAP90 has been reported to have an activity of clustering shaker-type K\(^{+}\) channels (11) and NMDA receptor 2B (NR2B) (12). The PDZ domains of PSD-95/SAP90 were found to interact with the C terminus (Ser/Thr)-X-Val (ST/XV) motif of the shaker-type K\(^{+}\) channel and NR2B, resulting in a clustering of ion channels and neurotransmitter receptors on the plasma membrane. Similarly, the PDZ domains of NE-dlg/SAP102 were shown to interact with the C terminus (ST/XV) motif of NR2B (7) and tumor suppressor protein APC (adenomatous polyposis coli) (6), suggesting that NE-dlg/SAP102 also contributes to a clustering of the diverse molecules in certain regions of the cells. In addition, the PDZ domain of neuronal nitric oxide synthetase was reported to interact with the PDZ domains of PSD-95/SAP90 and chapsyn-110/PSD-93 (13), suggesting that MAGUK proteins compose a heteromeric oligomer by means of their PDZ domains.

Various proteins that are involved in intracellular signaling systems have one or more SH3 domains; an SH3 domain is a protein-protein interaction module conferring a specific binding ability with a proline-rich motif in a target protein (14–17). However, no specific binding protein interacting with the SH3 domain of the MAGUK family has been identified yet. The GUK-like domains of PSD-95/SAP90, NE-dlg/SAP102, and chapsyn-110/PSD-93 have been recently reported to interact with a novel family of proteins called SAP-associated proteins (SAPAPs) (18–20). Although the function of SAPAPs has not been clarified yet, this finding suggests that the GUK-like domains of MAGUK family proteins also contribute to the protein-protein interaction.

We previously reported that NE-dlg/SAP102 is highly expressed in brain and endocrine tissues, such as pancreas, thym...
roid, and trachea (6). This tissue-specific expression suggests that NE-dlg/SAP102 plays specific roles in these neuronal and endocrine tissues. In the neurons, we found that NE-dlg/SAP102 is abundantly expressed in axons and dendrites of matured neurons (6). Muller et al. (7) also reported that the increase in NE-dlg/SAP102 mRNA levels parallels that of newly formed synapses in the developing rat cerebral cortex. Therefore, it has been speculated that NE-dlg/SAP102 contributes to the synapse formation by clustering neurotransmitter receptors and other molecules at synaptic sites. However, the molecular mechanisms responsible for the regulation of clustering and maintenance of the receptors at synaptic sites are largely unknown.

In the present study, we have first shown that NE-dlg/SAP102 protein colocalizes closely with NR2B at synaptic sites in cultured hippocampal neurons and increases in amount during postnatal development. To elucidate the involvement of NE-dlg/SAP102 in synapse formation, we searched for proteins that bind to NE-dlg/SAP102 by two-hybrid screening. We have found that calmodulin binds to the region containing the SH3 domain of NE-dlg in a calcium-dependent manner. A calmodulin-binding region of NE-dlg/SAP102 is predicted to form a basic amphiphilic α-helix, which was reported as a calmodulin-binding structure. Moreover, we have identified interaction between NE-dlg/SAP102 and PSD-95/SAP90 by two-hybrid screening, and this interaction was observed in the presence of both calmodulin and Ca2+. Our findings indicate that the association between NE-dlg/SAP102 and PSD-95/SAP90 is modulated by a calcium signaling pathway, and this mechanism may regulate the clustering of neurotransmitter receptors, resulting in formation and/or structural change in central synapses.

**EXPERIMENTAL PROCEDURES**

**Neonatal Rat Hippocampal Cell Culture**—Neonatal rat hippocampal cell cultures were prepared according to the method described previously (21). For the Western blot analysis, cells were harvested at the indicated days (Fig. 1A) in culture and lysed in SDS sample buffer. The samples were resolved by 10-20% SDS-polyacrylamide gel electrophoresis. After being blotted on a nitrocellulose filter, the filter was blocked with phosphate-buffered saline containing 10% skim milk for 1 h at room temperature and then incubated with anti-NE-dlg polyclonal antibody and anti-mouse IgG (Cappel, Aurora, OH) as the first antibody, and then fluorescein-conjugated goat anti-rabbit polyclonal antibody and anti-NMDA receptor 2B monoclonal antibody (Cappel, Nagoya, Japan) for a primary antibody, anti-mouse horseradish peroxidase (Amersham Pharmacia Biotech) for a secondary antibody and ECL (Amersham Pharmacia Biotech) for visualization.

**Surface Plasmon Resonance Measurements**—Calmodulin was purified from bovine brain as described previously (26). All of reagents and sensor chip for BIAcore instrumentation were purchased from BIAcore AB (Uppsala, Sweden). The specific interactions were analyzed by Surface Plasmon Resonance responses in BIAcore instrument (BIAcore AB). Four flow cells are placed in a surface of sensor chip CM5. Calmodulin was immobilized to a flow cell of sensor chip in order to be made as blank control and measure the nonspecific responses of analyte. GST-NE-dlg-(III) (analyte) was injected in both the control cell and to the calmodulin-immobilized cell at the flow rate of 6 μl/min in the running buffer (10 mM CaCl2, 10 mM DTT, 10 mM Tris (pH 7.5), NaCl 0.1 M) containing the indicated amount of CaCl2 or EGTA at 4 °C for 1 h. Calmodulin-agarose was immobilized by brief centrifugation at 4 °C, separated from the supernatant, and washed three times with 100 μl of binding buffer containing the same concentration of CaCl2 or EGTA as the binding reaction. The reactions were performed in the supernatant and bound to the calmodulin-agarose were resolved by SDS-polyacrylamide gel electrophoresis analysis, transferred to a nitrocellulose membrane and detected using anti-GST monoclonal antibody (MBL, Nagoya, Japan) for a primary antibody, anti-mouse horseradish peroxidase (Amersham Pharmacia Biotech) for a secondary antibody and ECL (Amersham Pharmacia Biotech) for visualization.
saline and lysed with 500 μl of extraction buffer (10 mM Tris-HCl at pH 7.5, 300 mM NaCl, 2% Triton X-100, 1 mM DTT, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.1 mM leupeptin, 1 μM pepstatin A, 54 μg/ml aprotinin) for 10 min on ice. After centrifugation at 12,000 × g for 20 min, the supernatant was diluted with equal volume of dilution buffer (10 mM Tris-HCl at pH 7.5, 2% Triton X-100, 1 mM DTT, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, 0.1 mM leupeptin, 1 μM pepstatin A, 54 μg/ml aprotinin). Fifty μl of GSH-agarose was incubated with 90 μg of GST or GST-PSD-95 (C-terminal) for 3 h, washed with washing buffer (10 mM Tris-HCl at pH 7.5, 150 mM NaCl, 2% Triton X-100, 1 mM DTT) three times, and divided into four tubes. The cell lysate, supplemented with CaCl2 or EGTA at a concentration of 1 mM and with or without calmodulin at a concentration of 3 μM, was added to each tube, conjugated for 2 h at 4 °C, and washed three times with washing buffer supplemented with 1 mM CaCl2 or EGTA. The proteins bound to the GSH-agarose were analyzed by Western blotting by using anti-hemagglutinin epitope antibody (12CA5) as described previously (6).

RESULTS

Expression of NE-dlg/SAP102 in Cultured Neurons—To investigate the role of NE-dlg/SAP102 in neurons, we first analyzed the expression of NE-dlg/SAP102 in the neonatal rat hippocampal cell culture by Western blot analysis (Fig. 1). NE-dlg/SAP102 protein, as well as NR2B, expressed at very low level until the 7th day in culture. However, the expression levels of both NE-dlg/SAP102 and NR2B began to elevate from the 10th day and reached a maximum at around 14 days of culture. These elevation of NE-dlg/SAP102 and NR2B expression during neuronal development appears to parallel the synapse formation in this neonatal hippocampal cell culture system (27). Next, we examined the subcellular localization of NE-dlg/SAP102 and NR2B in the rat hippocampal cells (21 days in culture). The antibody against NR2B gave punctate immunoreactivity along the dendrites (Fig. 2A), and the anti-NE-dlg/SAP102 antibody also gave a similar punctate pattern along the dendrites with diffuse staining in the cytoplasm of neurons (Fig. 2B). As shown in Fig. 2C, NE-dlg/SAP102 colocalized closely with NR2B in dendritic spines, at presumed synaptic sites. These findings suggest that NE-dlg/SAP102 and NR2B accumulate together at the synaptic sites during synapse formation in neuronal cells.

Identification of Interaction between NE-dlg/SAP102 and
Calmodulin—To obtain further insights into the action of NE-dlg/SAP102 on the synapse formation, we have tried to find novel NE-dlg/SAP102-interacting molecules by a yeast two-hybrid method using a human brain cDNA library as the prey. We used a region of amino acids 126–583, which contains the three PDZ domains and SH3 domain of human NE-dlg/SAP102 as the bait. Previously, we identified interaction between APC and NE-dlg by a two-hybrid screening using the same bait and a human fetal brain cDNA library as the prey (6). In the present study, we screened 10^6 clones and identified one positive clone that included a part of calmodulin cDNA encoding the region from the 14th to 142nd amino acid. This region contains all four calcium binding segments of calmodulin and lacked only a part of helices I and VII (28).

To identify the calmodulin binding site in NE-dlg/SAP102 protein, we performed a solution binding assay using calmodulin-agarose and a GST-fusion protein containing 32 amino acids corresponding to this basic α-helix (GST-NE-dlg-(IV)). GST-NE-dlg-(IV) showed binding ability to calmodulin-agarose in a Ca^{2+}-dependent manner when the washing was performed with 300 mM NaCl instead of the 150 mM NaCl used in the standard reaction (Fig. 3D, b). This result suggests that the binding ability of the 32 amino acids α-helical region of NE-dlg to calmodulin is also Ca^{2+}-dependent.

We performed surface plasmon resonance measurements in order to evaluate the specificity of the interaction between NE-dlg/SAP102 and calmodulin. GST-NE-dlg-(III) produced a dose-dependent increase of the apparent binding rate (dRU/s)
to the calmodulin-immobilized sensor chip (Fig. 4A). The apparent binding rate was proportional to the concentration of fusion protein (Fig. 4B). Analysis of the association and dissociation phases of these sensorgrams yielded an overall dissociation constant, $K_D$, of 44 nM ($k_a = 1.57 \times 10^4 \text{M}^{-1} \text{s}^{-1}$ and $k_d = 6.94 \times 10^2 \text{s}^{-1}$). Soluble calmodulin was coinjected with GST-NE-dlg as the competitor against the immobilized calmodulin (Fig. 5, A and C, b). The relative binding rate was calculated by dividing the binding rate in the presence of the competitor by that in the absence of the competitor. Because the amount of immobilized calmodulin was 77.8 fmol and the volume of the flow cell was 60 nl, the average concentration of immobilized calmodulin in the flow cell was 1.3 µM. It is noteworthy that the concentration at which the competitor inhibits 50% of binding, IC$_{50}$, of soluble calmodulin (1.6 µM) is very close to the average concentration of immobilized calmodulin. In contrast, the IC$_{50}$ of soybean trypsin inhibitor, which has a molecular weight and acidity similar to those of calmodulin, was more than 10-fold higher than immobilized calmodulin (Fig. 5, B and C, a). These results clearly indicate that NE-dlg/SAP102 specifically binds to calmodulin.

**Effect of Calmodulin Binding on Interaction between NE-dlg/SAP102 and (S/T)XV-COOH Motif**—We examined whether the interaction of calmodulin with NE-dlg/SAP102 affects the binding between PDZ domains of NE-dlg/SAP102 and the (S/T)XV-COOH sequence by using surface plasmon resonance measurement. The biotinylated peptide PEP7154 (biotin-NGHVYEKLSSIESDV-COOH) corresponding to the C-terminal region of rat NR2B was first immobilized on the sensor chip, and GST-NE-dlg-(I) protein and calmodulin were coinjected into the flow cell. PEP7153 (biotin-NGHVYEKLSSIESD-COOH), which lacks the C-terminal Val residue, was used for the negative control experiment. GST-NE-dlg-(I) bound to the immobilized NR2B peptide but not to the peptide lacking the C-terminal Val residue (data not shown), and the coinjection with calmodulin did not suppress but, rather, barely increased the binding rate of GST-NE-dlg-(I) to the NR2B peptide (Fig. 6). This finding suggests that calmodulin binding...
Fig. 5. Competitive inhibition of the interaction between GST-NE-dlg and immobilized calmodulin by soluble calmodulin. A, sensorgrams of GST-NE-dlg-(III) (0.25 μM) coinjected into the calmodulin-immobilized sensor chip with soluble calmodulin at the following concentrations: a, 0 μM; b, 0.25 μM; c, 0.75 μM; d, 2.5 μM; e, 7.5 μM; f, 25 μM. B, sensorgrams of 0.25 μM GST-NE-dlg-(III) coinjected to the calmodulin-immobilized sensor chip with soybean trypsin inhibitor at the following concentrations: a, 0 μM; b, 0.25 μM; c, 2.5 μM; d, 25 μM. C, relative binding rate of NE-dlg to immobilized calmodulin was suppressed by addition of soluble calmodulin. a, soybean trypsin inhibitor; b, soluble calmodulin. The apparent binding rate (dRU/s) was calculated from the bold areas of each sensorgram shown in A and B. The relative binding rate was calculated as indicated under “Experimental Procedures.”
Fig. 6. Calmodulin did not affect the interaction between GST-NE-dlg and NR2B peptide. GST-NE-dlg-(I) (25 nM) was coinjected into the NR2B peptide-immobilized sensor chip with calmodulin at the following concentrations: a, 0 μM; b, 1 μM; c, 3 μM; d, 10 μM. GST (500 nM) (e) was injected into the NR2B peptide-immobilized sensor chip as a negative control.

Identification of Interaction between NE-dlg/SAP102 and PSD-95/SAP90—To elucidate the biological significance of NE-dlg/calmodulin interaction in a neuronal cell, we attempted to identify another cellular protein that binds to the region near the calmodulin binding site of NE-dlg by two-hybrid screening using a human brain cDNA library as the prey. Among 1.6 × 10^6 clones screened, a positive clone contained a 1.7-kilobase pair insert, the sequence of which was identical to a part of human PSD-95/SAP90 cDNA. The insert encoded the C-terminal 284-amino acid fragment of the PSD-95/SAP90 protein. This region contains a part of SH3 domain and an entire GUK-like domain. To test whether calmodulin binding can modulate interaction between NE-dlg/SAP102 and PSD-95/SAP90, we performed a pull down assay. Whole cell lysate of COS-7 cells transfected with pCGN-NE-dlg-ΔGUK were incubated with either GST or the GST-PSD-95 (C-terminal) bound GSH-beads. In the presence of both Ca^{2+} and calmodulin, NE-dlg-ΔGUK was strongly associated with PSD-95 (C-terminal) but not with GST (Fig. 7, lanes 3 and 4). However, specific interaction between NE-dlg-ΔGUK and GST-PSD-95 was not observed in the presence of calmodulin alone (Fig. 7, lanes 5 and 6) or Ca^{2+} alone (Fig. 7, lanes 7 and 8). These results indicate that NE-dlg/SAP102 specifically interact with PSD-95/SAP90 in the presence of Ca^{2+} and calmodulin.

DISCUSSION

In this study, we have shown that NE-dlg/SAP102 and NR2B colocalize at putative synapses in cultured rat hippocampal neurons and that their expression are coincident with synapse formation. Because the expression of PSD-95/SAP90 protein is also known to parallel CNS synaptogenesis (11), the clustering of neurotransmitter receptors by multiple MAGUK proteins may play a role in the synaptic architecture formation. To understand the mechanisms that underlie the clustering and localization of membrane-associated proteins in neurons, we performed two-hybrid screening using a human brain cDNA library to identify NE-dlg/SAP102-interacting proteins. Initially, we identified the interaction between NE-dlg/SAP102 and calmodulin. The in vitro solution binding assay using calmodulin-agarose showed that a binding site for calmodulin lies at the basic amino acid-rich region that flanks the SH3 motif of NE-dlg/SAP102. The surface plasmon resonance measurement revealed that the interaction between calmodulin and NE-dlg/SAP102 was specifically inhibited by the addition of soluble calmodulin in the system.

Although the calmodulin-binding regions of the target proteins have not shown a rigid consensus sequence, many of them are known to possess a region that is characterized by a basic amphipathic α-helix consisting of approximately 20 amino acids (29, 30). The calmodulin-binding region of NE-dlg/SAP102 also contains basic amino acids interspersed among hydrophobic residues and is predicted to form a basic amphipathic α-helix based on the present computational analysis and α-helix modeling. Moreover, the positions of the basic amino acids in this region are mostly conserved among hdlg-1/SAP97, chapsyn-110/PSD-93, PSD-95/SAP90, and Drosophila DLG-1, suggesting that calmodulin may be able to interact with all of these proteins (Fig. 8).

The PDZ domains of NE-dlg/SAP102 are known to interact tightly with (S/T)XV motifs located on the C terminus of NMDA receptor and APC tumor suppressor protein. The x-ray structure of the third PDZ domain of hdlg-1/SAP97 alone (31) and PSD-95 binding to a 9-mer peptide (32) revealed that the C-terminal hydrophobic side chain is buried deeply in a hydrophobic pocket of the PDZ domain and that the C-terminal carboxylate interacts with the GLGF motif, which is highly conserved in most of the PDZ domains. Using a peptide library approach, Songyang et al. (33) showed that the PDZ domain of hdlg or PSD-95 could selectively bind octapeptides having a consensus sequence of (S/T)XVVI at the C terminus. In the present study, we have tried to determine whether calmodulin can modulate the interaction between the PDZ domains and (S/T)XV motif. When calmodulin and GST-NE-dlg were applied simultaneously to the NR2B-peptide immobilized sensor chip, the binding rate of GST-NE-dlg was not affected by the addition of calmodulin. This finding suggests that calmodulin does not negatively regulate the interaction between NE-dlg/SAP102 and the (S/T)XV motif.

We have found that another MAGUK protein, PSD-95/SAP90, also specifically interacts with the region flanking the SH3 motif of NE-dlg protein in the presence of both Ca^{2+} and calmodulin. It is noteworthy that the binding of NE-dlg/SAP102 and PSD-95/SAP90 is not mediated by their PDZ domains. The two-hybrid screening demonstrated that SH3 and intervening sequences of NE-dlg/SAP102 is associated with a
part of SH3 and GUK-like domain of PSD-95/SAP90. Because both NE-dlg/SAP102 and PSD-95/SAP90 can bind to the C terminus of NR2B by their PDZ domains, our finding suggests that heteromeric complex formation of MAGUK proteins contributes clustering of NR2B at postsynaptic membrane.

Ca\(^{2+}\)/calmodulin has been reported to bind NMDA receptor type 1 (NR1) and to disrupt the interaction between \(\alpha\)-actinin and NR1 (34, 35). Wyszynski et al. (35) suggested that a postsynaptic Ca\(^{2+}\) influx, perhaps through activated NMDA receptors, may lead to a Ca\(^{2+}\)-dependent detachment of NMDA receptors from the actin cytoskeleton and their redistribution during synaptic activity (35). This Ca\(^{2+}\)/calmodulin-dependent detachment can liberate the NMDA receptor from the anchor of \(\alpha\)-actinin and facilitate the clustering of neighboring receptors through the binding of PSD-95/SAP90 and NE-dlg/SAP102. Taken together, it can be speculated that a Ca\(^{2+}\) entry from NMDA receptors may modulate interaction among NE-dlg/SAP102, PSD-95, and NMDA receptors, and the redistribution of those molecules may consequently play an important role in the assembly of the synapses (Fig. 9).

There is considerable evidence supporting a functional involvement of MAGUK proteins, NMDA receptor, and Ca\(^{2+}\) signaling in the synaptogenesis (36). Mutation of the DLG-1 gene was shown to disrupt the synaptic morphology of the Drosophila neuromuscular junction (37). Furthermore, the degree of axonal sprouting in cultured young Xenopus neurons is increased by NMDA receptor blockade, and this effect can be mimicked by chelating Ca\(^{2+}\) (38). This finding suggests that NMDA receptor activation may suppress inappropriate growth of axons so that they can proceed with subsequent stages of appropriate synaptogenesis (39, 40). This evidence, together with our findings, indicates that Ca\(^{2+}\)/calmodulin-dependent regulation of NMDA receptor clustering mediated by the neuronal MAGUK proteins may serve as an initial step for synaptogenesis.

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