Identification of a Potential Tyrosine Phosphorylation Site on the NR2B Subunit of the N-methyl-D-aspartate Receptor

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Abstract

The 2B subunit of N-methyl-D-aspartate (NMDA) receptors (NR2B) is the major phosphotyrosine-containing protein in the postsynaptic density (PSD). In order to identify the site for tyrosine phosphorylation on NR2B, a mass spectrometry was applied on tryptic and endolys-C peptides. The NR2B subunit was isolated from N-octyl glucoside (N OG)-insoluble PSD fraction through SDS-PAGE and Electroelution. The eluted protein was confirmed to be NR2B and phosphorylated on tyrosine by its cognate antibody and phosphotyrosine-specific antibody. By matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry of the peptides generated by digesting the eluted NR2B with trypsin or endolys-C, a potential site for tyrosine phosphorylation could be identified as Tyr-1304.

Key words : PSD, NMDA receptor, NR2B, Tyrosine phosphorylation, MALDI-TOF

Introduction

Synaptic plasticity such as long-term potentiation (LTP) and long-term depression (LTD) is the synaptic basis for learning and memory. The N-methyl-D-aspartate receptors, which are ligand and voltage-gated Ca²⁺ channels, play a critical role in the induction of both LTP and LTD. The NMDA receptors are composed of two classes of subunits in hetero-oligomeric associations, the NR1 subunit and the regulatory NR2(A-D) subunits. The NR2A and NR2B are the major regulatory subunits of NMDA receptors in the forebrain. The NR2B is phosphorylated by both calcium/calmodulin-dependent protein kinase II (CaMKII), which is a serine/threonine kinase, and protein-tyrosine kinase (PTK). Interestingly, the NR2B is the major phosphotyrosine-containing protein in the postsynaptic density (PSD). Moreover, after induction of LTP, there is an increase in the tyrosine phosphorylation of NR2B. These findings indicate that the tyrosine phosphorylation of NR2B is important for the regulation of the receptor function. However, direct demonstration of the role of tyrosine phosphorylation of the receptor requires identification of sites on the receptor subunits. Here, I report the identification of a potential site of in vivo tyrosine phosphorylation of the NR2B subunit.

Materials and Methods

Isolation and fractionation of the PSD fraction
The rat (Sprague-Dawley) forebrain PSD fraction was prepared by washing synaptosome-enriched fraction with 0.5% Triton X-100 as described. The PSD fraction was extracted with detergents at 4°C for 30 min.

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with stirring and the soluble and insoluble fractions were separated by centrifugation at 200,000×g for 30 min at 4°C.

Immunoblot
Proteins were transferred to nitrocellulose membrane and incubated with anti-NR2B antibody\(^9\) or anti-phosphotyrosine monoclonal antibody (clone 4g10, Upstate Biotechnology Inc.) as described previously\(^2\) and the bands were visualized with alkaline phosphatase-conjugated secondary antibodies.

Purification of NR2B
The NOG-pellet fraction were electrophoresed in 6% SDS-polyacrylamide gels. After staining the gels with 0.2% (w/v) Coomassie blue R-250 (BioRad) in 10 mM Tris-Cl (pH8.0) and electroeluted from the gel pieces with an Elutrap device (Schleicher & Schuell) as described\(^9\).

Digestion with trypsin and endolys-C
The electroeluted NR2B was electrophoresed in a 6% SDS-gel and briefly stained with Coomassie dye to visualize the band. The protein bands were excised and digested with trypsin and endolys-C as described previously\(^9\).

Mass spectrometry
Mass spectrometry was performed by the Protein/Peptide Micro Analytical Laboratory at Caltech with a Perseptive Biosystems/Vestec Lasertech II reflector for matrix-assisted, laser desorption ionization, time-of-flight mass spectrometry (MALDI-TOF). Peptides were mixed with an a-cyano-4-hydroxycinnamic acid matrix solution, dried, and placed in the mass spectrometer. Data were collected in both linear and reflector modes.

Results and Discussion

In this report, the NR2B subunit was isolated from NOG-insoluble PSD fraction through SDS-PAGE and electroelution. The eluted NR2B was specifically recognized by its cognate antibody and phosphotyrosine-specific antibody, indicating that the eluted protein is the NR2B and that the phosphorylation on tyrosine residue is preserved throughout the isolation procedure. By mass spectrometry of the peptides generated by digesting with trypsin or endolys-C, the site for tyrosine phosphorylation could be identified as Tyr-1304.

The electroeluted NR2B retains its phosphorylation on tyrosine
The PSD is a large protein complex which is readily seen on electron microscope of the mammalian central nervous system (CNS) synapse\(^3,4\). An electron microscopic observation of cerebellar PSD using freeze-fracture techniques showed that the PSD is composed of smaller round modules associated with cytoskeleton\(^4\). Since the proteins in PSD are mostly hydrophobic, the methods for purification of its component proteins are very limited. In this work, I used SDS-PAGE to separated the NR2B protein band from other proteins in the PSD and the NR2B was electroeluted. Usually, the efficiency for protein electroelution is low. However, by omitting a fixative agent in the Coomassie staining solution, the elution efficiency could be enhanced greatly. In Coomassie staining, the eluant revealed only one band with the same molecular size as NR2B (Fig. 1), and the band intensity in the lane that is applied with the relatively same volumes of the initial PSD and eluant fractions was quite similar (Fig. 1D). A densitometric analysis revealed that the elution efficiency was approximately 70% (not shown). When the eluted protein was immunoblotted with NR2B-specific antibody, there appeared a single strong signal associated with the protein, indicating that the eluted protein is NR2B. In order to verify that the eluted NR2B is still phosphorylated on tyrosine, which is phosphorylated in vivo, an immunoblot analysis was carried out with a NR2B-specific anti-
body. As shown in Fig. 1C, the eluted NR2B was recognized by the antibody strongly, indicating that the phospho group on tyrosine is well preserved throughout the isolation procedure. These results confirm that the protocol used in this work is a reliable method for isolating a PSD protein with phosphorylation of tyrosine.

Yr-1304 is phosphorylated.

Protein-tyrosine phosphorylation has been suggested to play an important role in synaptic transmission. However, the role of tyrosine phosphorylation in the modulation of synaptic function in CNS synapses is still unclear. NMDA receptors play a critical role in synaptic plasticity such as LTP and LTD. The PSD-associated NR2B was shown to be the major tyrosine-phosphorylated protein in the PSD. However, the site for tyrosine phosphorylation is not identified. In this work, I attempted to find the site for tyrosine phosphorylation on NR2B by MALDI-TOF mass spectrometry in both the linear and reflector modes. In the reflector mode, fragmentation of the phospho group on phosphopeptides usually produces a new peptide peak with a mass 80 atomic mass units less than that of the phosphopeptide itself when the peptide is phosphorylated on tyrosine. Two potential tyrosine phosphorylated peptides of NR2B could be identified (Table 1). A signal with a molecular mass 1985.7, found in the MALDI-TOF with a peptide mixture digested with endolys-C, matched well with LRRQHSDTFVSDLQK which corresponds to amino acid positions from 1298 to 1312 of NR2B. The tyrosine-unphosphorylated and -phosphorylated molecular mass of the peptide are calculated to be 1906.13 and 1986.13, respectively. The molecular mass found in MALDI-TOF was close to the phosphorylated peptide. This site for tyrosine phosphorylation was further confirmed by tryptic peptides. There appeared a peak with molecular mass 1559.3 on a linear mode (Fig. 2B). This molecular mass was well matched with QHSYDTFVSDLQK which correspond to the position 1301-1312 of NR2B. The molecular masses for the unphospho- and phospho-peptides are 1479.70 and 1559.79, respectively. Therefore, the signal in MALDI-TOF is indicated to be tyrosine-phosphorylated QHSYDTFVSDLQK. This conclusion is further augmented on reflector mode. When the tryptic peptides were analyzed by a reflector mode, there appeared a new peak with a molecular mass 1479.4 (Fig. 2C, arrowhead) in addition to the parent 1559.4 peak. Since the reflector mode generates new peaks with molecular masses sma-
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Table 1. Phosphotyrosine-containing peptides of NR2B.

<table>
<thead>
<tr>
<th>enzyme used</th>
<th>position in NR2B</th>
<th>peptide sequence</th>
<th>mass-1</th>
<th>mass-2</th>
<th>found</th>
</tr>
</thead>
<tbody>
<tr>
<td>trypsin</td>
<td>1301-1312</td>
<td>QHSYDTFVDLQK</td>
<td>1479.70</td>
<td>1559.79</td>
<td>1559.4</td>
</tr>
</tbody>
</table>

The mass-1 and mass-2 are atomic masses of the dephosphorylated and phosphorylated peptides on Tyr-1304, respectively.

Fig. 2. Identification of phosphotyrosine peptides by MALDI-TOF.
A. The electroeluted NR2B was digested with endolys-C and data were collected on a linear mode. The phosphopeptide was indicated as an arrow with the amino acid sequence.
B and C. The electroeluted NR2B was digested with trypsin and data were collected on both linear (B) and reflector modes (C). The phosphopeptides are indicated as arrows with amino acid sequences. A signal for the dephosphorylated peptides on reflector mode is indicated as an arrowhead in C.

Furthermore, a same sequence RQSHYD is present in the NR2A, strongly suggesting that the NR2A may also be phosphorylated on serine and tyrosine in this peptide, if the NR2A is phosphorylated on tyrosine. The effects of the phosphorylation on tyr-1304 is not known. Recently, Rostas et al.\textsuperscript{10} reported that the tyrosine phosphorylation of NR2B increases after induction of LTP. However, it is not known if the LTP induces phosphorylation on tyr-1304, because the NR2B used in this work is isolated directly from the brain. Since the NR2B is phosphorylated on tyrosine in the early stage

Fig. 3. Tyrosine and serine phosphorylation sites for NR 2B.
The serine and tyrosine phosphorylation sites by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein tyrosine kinase (PTK), respectively, are shown with the amino acid positions in NR2B. A similar sequence found in NR 2A is shown below the NR2B.

of brain development, tyr-1304 may well be the developmentally phosphorylated site. Further studies are necessary to resolve this point.

References

초록: NMDA 수용체 아단위 2B의 Tyrosine 인산화 위치의 동정

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