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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 (NOG)-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[1]. The N-methyl-D-aspartate receptors, which are ligand and voltage-gated Ca^{2+} channels[2,3], play a critical role in the induction of both LTP and LTD[4,5,6], The NMDA receptors are composed of two classes of subunits in hetero-oligomeric associations, the NR1 subunit and the regulatory NR2(A-D) subunits[2,3], The NR2A and NR2B are the major regulatory subunits of NMDA receptors in the forebrain[2,3]. The NR2B is phosphorylated by both calcium/calmodulin-dependent protein kinase II (CaMKII), which is a serine/threonine kinase[7,8], and protein-tyrosine kinase (PTK)[9]. Interestingly, the NR2B is the major phosphotyrosine-containing protein in the postsynaptic density (PSD)[9]. Moreover, after induction of LTP, there is an increase in the tyrosine phosphorylation of NR2B[10]. 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[11]. The PSD fraction was extracted with detergents at 4℃ for 30 min

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

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[12] 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[13,14]. An electron microscopic observation of cerebellar PSD using freeze-fracture techniques showed that the PSD is composed of smaller round modules associated with cytoskeleton[14]. 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. 1.D). 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.

Tyr-1304 is phosphoryled.

Protein-tyrosine phosphorylation has been suggested to play an important role in synaptic transmission[15,16]. 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 the synaptic plasticity such as LTP and LTD[4,5,6]. The PSD-associated NR2B was shown to be the major tyrosine-phosphorylated protein in the PSD[9]. 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[17]. 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 phosphopepdide itself when the peptide is phosphorylated on tyrosine[17]. 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 LRRQHSYDTFVDLQK which corresponds to amino acid positions from 1298 to 1312 of NR2B (Fig. 2A). The tyrosine-unphosphorylated and -phosphorylaed 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 QHSYDTFVDLQK 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 QHSYDTFVDLQK. 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-

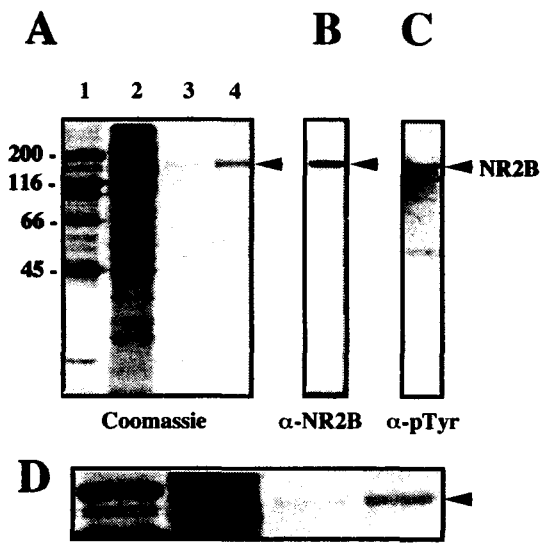

Fig. 1. Electroelution of tyrosine phosphorylated NR2B. A and D. Coomassie staining of the electroeluted protein. The PSD was isolated from rat forebrain and washed with 1% NOG (lane 1). The NOG-insoluble fraction was electrophoresed in 6% SDS-gels and the 180 kDa band was electroeluted as described in Materials and Methods. The eluted protein was applied in a 8% SDS-gel for a relatively same (lane 3) or double (lane 4) of the initial PSD volume. An enlarged image was shown in D.
B and C. Immunoblots. The eluted protein was immunoblotted with an antibody specific for NR 2B (B) or phosphotyrosine (C). The position of NR2B is indicated as arrowheads. Molecular size markers (lane 1) is shown in left in kilodaltons.

Table 1. Phosphotyrosine-containing peptides of NR2B.

enzyme used	position in NR2B	peptide sequence	mass-1	mass-2	found
trypsin	1301-1312	QHSYDTFVDLQK	1479.70	1559.79	1559.4
endolys-C	1298-1312	LRRQHSYDTFVDLQK	1906.13	1986.13	1985.7

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

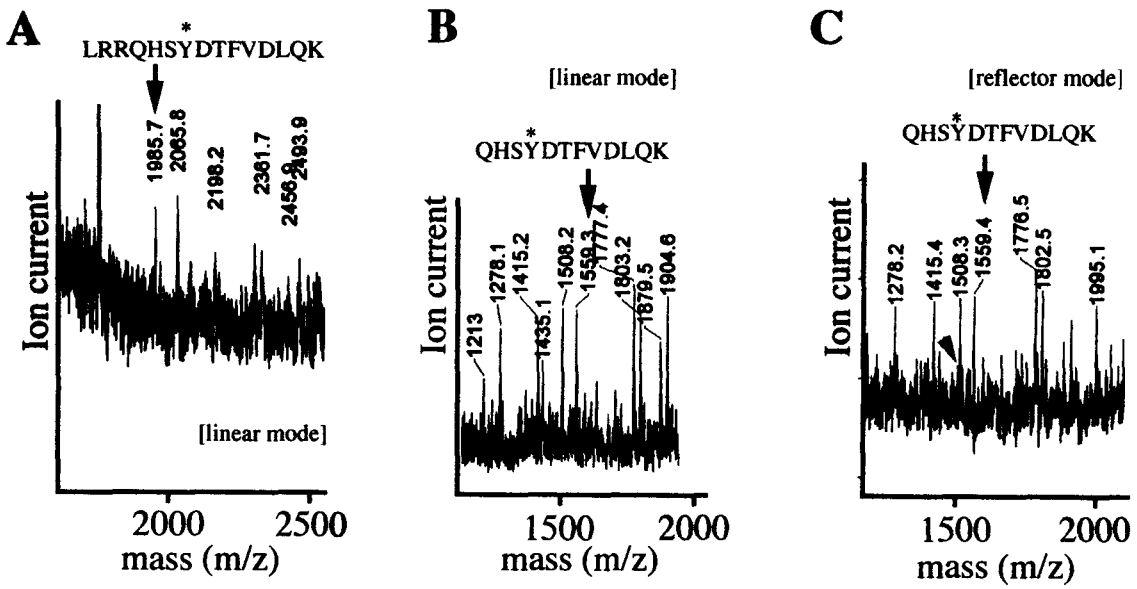

Fig. 2. Identification of phosphotyrosine peptides by MALDI-TOF.
A. The electroeluted NR2B was digested with endolys-C and data were collected on a linear mode. The phosphopeptide was indicated as an arrow with the amino acid sequence.
B and C. The electroeluted NR2B was digested with trypsin and data 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.

ller than the parent ones by 79 in serine phosphorylation and 80 in tyrosine phosphorylation, this result confirms that the phosphorylation site on this peptide is Tyr-1304.

The NR2B is highly regulated by both CaMKII and PTK. Recently Omkumar *et al.*[7] identified Ser-1303 as a major phosphorylation site for CaMKII on NR2B. Interestingly, this serine is positioned just in front of the tyrosine which is found to be phosphorylated in this work (Fig. 3). These results indicate that this region of the NR2B is under intensive regulation by protein kinases. 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.*[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

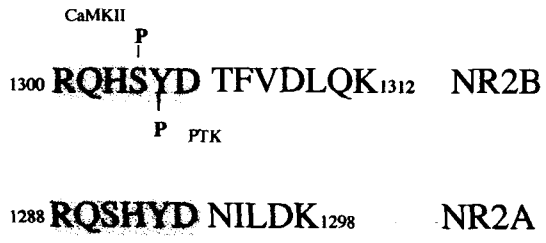

Fig. 3. Tyrosine and serine phosphorylation sites for NR 2B.
The serine and tyrosine phosphorylation sites by calcium/calmodulin-dependent protein kinase II (CaMKII) and protein tyrosine kinase (PTK), respectively, are shown with the amino acid positions in NR2B. A similar sequence found in NR 2A is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

of brain development[18], tyr-1304 may well be the developmentally phosphorylated site. Further studies are necessary to resolve this point.

References

1. Squire, L. R. : Memory and the hippocampus : a synthesis from findings with rats, monkeys, and humans. *Psychological Rev.* 99, 195−231(1992).
2. Ishii, T., Moriyoshi, K., Sugihara, H., Sakurada, K., Kadotani, H., Yokoi, M., Akazawa, C., Shigemoto, R., Mizuno, N., Masu, M., and Nakanish, S. : Molecular characterizartion of the family of the N-methyl-D-aspartate receptor subunits. *J. Biol. Chem.* 268, 2836−2843(1993).
3. Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakman, B., and Seeburg, P. H. : Heteromeric NMDA receptors : molecular and functional distinction of subtypes. *Science* 256, 1217−1221(1992).
4. Bliss, T. M. V., and Collingridge, G. L. : A synaptic model of memory : long-term potentiation in the hippocampus. *Nature* 361, 31−39(1993).
5. Ito, M. : Long-term depression. *Ann. Rev. Neurosci.* 12, 85−102(1989).
6. Linden, D. J. : Long-term synaptic depression inthe mammalian brain. *Neuron* 12, 457−472(1994).
7. Omkumar, R. V., Kiely, M. J., Rosenstein, A. J., Min, K.-T., and Kennedy, M. B. : Identification of a phosphorylation site for calcium/calmodulin-dependent protein kinase II in the NR2B subunit of the N-methyl-D-aspartate receptor. *J. Biol. Chem.* 271, 31670−31678(1996).
8. Moon, I. S., Jin, D. H., and Ko, B. H. : Phosphorylation of N-methyl-D-aspartate receptor subunit 2B by Ca2%/calmodulin-dependent protein kinase II. Mol. Cells 5, 475−480(1995).
9. Moon, I. S., Apperson, M. L., and Kennedy, M. B. : The major tyrosine-phosphorylated protein in the postsynaptic density fraction is N-methyl-D-aspartate receptor subunit 2B. *Proc. Natl. Acad. Sci. USA* 91, 3954−3958(1994).
10. Rostas, J. A., Brent, V. A., Voss, K., Errington, M. L., Bliss, T. V., and Gurd, J. W. : Enhanced tyrosine phosphorylation of the 2B subunit of the N-methyl-D-aspartate receptor in long-term potentiation. *Proc. Natl. Acad. Sci. USA* 93, 10452−10456(1996).
11. Carlin, R. K., Grab, D. J., Cohen, R. S., and Siekevitz, P. : Isolation and characterization of postsynpatic densities form various brain regions : enrichment of different types of postsynaptic densities. *J. Cell Biol.* 86, 831−843(1980).
12. Cudmore, S. B., and Gurd, J. W. : Postnatal age and protein tyrosine phsphorylation at synapses in the developing rat brain. *J. Neurochem.* 57, 1240−1248 (1991).
13. Kennedy, M. B. : The postsyanptic density. Curr. Opinions Neurobiol. 3, 732−737(1993).
14. Landis, D. M. D., Reese, T. S., and Raviola, S. : Differences in membrane structure between excitatory and inhibitory components of the reciprocal synapse in the olfactory bulb. *J. Comp. Neurol.* 155, 67−92 (1974).
15. Huganir, R.L., and Greengard, P. : Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron* 5, 555−567(1990).
16. Hunter, T. : Protein kinases and phosphatases : the Yin and Yang of protein phosphorylation and signaling. *Cell* 80, 225−236(1995).
17. Mann, M., and Wilm, M. : Electrospray mass spectrometry for protein characterization. *TIBS* 20, 218 −224(1995).
18. Jin, D. H., Jung, Y. W., Ham, S. H., Ko, B. H., and Moon, I. S. : Developmental expression, subcellular localization, and tyrosine phosphorylation of NR2A and NR2B in the rat brain. *Mol. Cells* 7, 64−71 (1997).

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초록 : NMDA 수용체 아단위 2B의 Tyrosine 인산화 위치의 동정

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NR2B 는 연접후 치밀질의 주요 tyrosine 인산화 단백질이다. 본 연구에서는 mass spectrometry 방법을 적용하여 NR2B의 tyrosine 인산화 위치를 동정하였다. NR2B를 N-octyl glucoside (NOG)에 용해되지 않는 PSD 분획으로부터 SDS-PAGE와 electroelution방법으로 분리하였다. 분리한 단백질을 NR2B와 phosphotyrosine에 특이한 항체로 조사한 결과 이들은 phosphotyrosine을 유지하고 있는 NR2B임이 확인되었다. 이 단백질을 trypsin 혹은 endolys-C 처리하고, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry 방법으로 조사한 결과 Tyr-1304이 인산화됨을 확인하였다.