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Studies on the phosphotyrsine-proteins in the rat cerebellar PSD fraction

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Abstract

The signal transduction through tyrosine kinases play important roles in neuronal development and synaptic regulation. We carried out immunoblot analyses to study tyrosine-phosphorylated proteins in the rat cerebellar postsynaptic density (PSD), a protein-rich cytoskeletal specialization underlying beneath the postsynaptic membrane. The overall protein composition of cerebellar PSD fractions was similar to that of the forebrain's and only a few bands were different in Coomassie stain. Immunoblot analyses with phosphotyrosine-specific antibody (4G10) showed that there are many more tyrosine-phosphorylated proteins in the cerebellar PSD than in the forebrain PSD. Interestingly, a major phosphotyrosine signals in cerebellar PSD fractions was associated with a 50 kD molecular size, named as PSD-50. Migration of PSD-50 coincided with that of αCaMKII and remained in the pellet fraction after N-octylglucoside extraction. These results indicate that tyrosine phosphorylation is important in cerebellar synaptic regulation and that the PSD-50 may be same as αCaMKII or a new protein which is a major substrate of tyrosine kinase.

Key words : *postsynaptic density, tyrosine phosphorylation, immunoblot*

Introduction

Synapses are special structures through which neurons communicate. In the central nervous system (CNS), the synaptic transmission is highly regulated and the efficacy of synaptic transmission can be modified depending on how a certain synapse is used. Tight regulation of synaptic signal transmission is essential for normal brain function and deregulation of this process underlies many neuronal diseases[1-2].

A prominent structure in the CNS synapse is the postsynaptic density (PSD). PSD is a protein-rich complex that tightly adheres to the postsynaptic membrane apposed to the active zone in the presynaptic membrane[3]. The PSD is especially well developed in excitatory CNS synapses[4]. Although the molecular composition of the PSD is very limited, several functions of the PSD were proposed including regulation of adhesion of pre- and postsynaptic membranes, receptor clustering and functions[5]. It is crucial, however, to identify the molecular components of the PSD in order to understand its function. Since biochemical methods for preparation of a PSD-enriched fraction were developed, immunoblot studies revealed that the cytoskeletal proteins tubulin, actin, and fodrin are among the most prominent proteins in the PSD fraction (see a review by Gurd[6]). A wide

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variety of regulatory enzyme activities have also been observed in the PSD fraction, including calmodulin-dependent cyclic nucleotide phosphatase and protein kinase activities, as well as cAMP-dependent protein kinase activity[5]. Recently, protein microsequencing and molecular cloning techniques have been imployed to identify PSD proteins. The first protein identified by these techniques was the subunit of the type II calcium/calmodulin-dependent protein kinase (CaMKII)[7-8]. The CaMKII is enriched in the core fraction and has been localized to the PSD by immunoelectron microscopy[8]. CaMKII mediates signal transduction in response to calcium influx at the synapse and is important for synaptic plasticity[9]. A second PSD protein identified was PSD-95[10], a novel brain-specific protein with significant homology to the Drosophila discs-large protein (dlg)[11]. It is also localized to the PSD by immunocytochemistry[12]. A third core PSD protein was the 2B subunit of the NMDA receptor (NR2B), which is the major tyrosine-phosphorylated protein in the PSD fraction[13]. Results so far reinforce that the PSD is a dynamic structure involved in synaptic regulation.

On the other hand, high levels of protein-tyrosine kinases in the CNS have suggested that tyrosine phosphorylation is involved in regulations of neural development and synaptic transmission[14]. Long-term potentiation (LTP), a persistent increase in synaptic efficacy, was blocked by tyrosine kinase inhibitors[15], and was impaired in fyn-deficient mutant mice[16]. Furthermore, activation of NMDA receptors stimulated protein tyrosine phosphorylation[17]. These results indicate that signal transduction through tyrosine phosphorylation is important in synaptic regulation and prompted us to search the target proteins downstream of tyrosine kinases. Here, we report that a major tyrosine-phosphorylated protein in cerebellar PSD fraction is tightly associated with PSD and migrates with αCaMKII.

Materials and Methods

Antibodies

The monoclonal antibody specific for phosphotyrosine [4G10] was purchased from Upstate Biotechnology (Lake Placid, New York, USA) and the monoclonal antibody for CaMKII was kindly provided by Dr. Mary Kennedy, California Institute of Technology, Pasadena, California.

Subcellular fractionation

The PSD fractions were prepared according to Carlin *et al.*[18] with some modifications. After decapitation, rat brains were isolated and the cerebellums were dissected out. Synaptosomes, prepared from cerebellar homogenate through discontinuous sucrose gradients, were treated with 0.5% Triton X-100 at 4oC for 15 min, and centrifuged at 36,800×g for 30 min at 4℃. The pellet [PSD fraction] was resuspended in 40 mM Tris-Cl (pH 8.0).

Extraction of PSD fraction with N-octylglucoside (NOG)

PSD fractions were adjusted to a final concentration of 1% NOG, and mixed at 4℃ for 30 min in a tumbler. The solution was centrifuged at 36,800×g for 30 min at 4℃ and the pellet fraction, resuspended in 40 mM Tris-Cl, pH8.0, and supernatant were stored at −70℃.

Immunoblots

After electrophoresis the proteins were transferred to nitrocellulose (NC) and immunoblotted according to Cudmore and Gurd[19] with phosphotyrosine-specific monoclonal antibody (4G10) (at 1 : 2,000 dilution) or an αCaMKII antibody. Color was developed by incubation with AP-conjugated secondary antibodies according to the supplier's instructions.

Analytical methods

Protein was assayed by the method of Peterson[20]. SDS-polyacrylamide gel electrophoreses were performed according to the method of Laemnli[21] and the proteins were visualized by staining with Coomassie R-250 (BioRad).

Results

Protein composition of the cerebellar PSD fraction

The PSD is a protein-rich complex. In order to find the protein components of the PSD, we isolated PSD fractions from the fresh rat cerebellum and separated in a 6% SDS-gel. About 0.5 mg of PSD could be obtained from 1 g of fresh cerebellum. The PSD represents 0.05 % of the brain tissue. Coomassie stain of the SDS-gel revealed about 50 distinct bands above smear background (Fig.2). The overall profile was similar to that of the forebrain PSD fractions (see Fig.2), and some protein bands such as fodrin, NMDA receptor subunits, hexokinase, actin, and CaMKII could be identified by molecular sizes and band intensities. However, the band intensity of CaMKII was significantly lower in cerebellar PSD than in forebrain PSD fraction (Fig.1 and Fig.2).

Tyrosine-phosphorylated proteins in the cerebellar PSD fraction

In order to understand and compare the composition

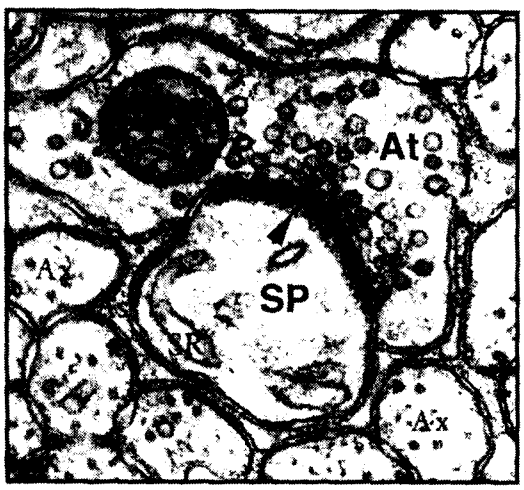

Fig. 1. An electron micrograph of a cerebellar synapse showing the postsynaptic density (arrowhead). At, axon terminal ; SP, spine. Adapted from Peters et al.[3].

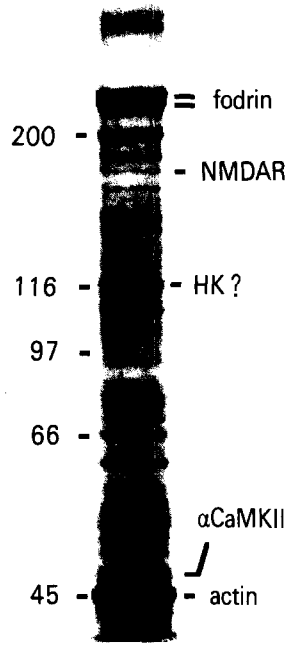

Fig. 2. An electrophoretic profile of cerebellar PSD proteins. The cerebellar PSD fraction (40 μg) was electrophoresed in a 6% SDS-gel and stained with Coomassie blue R-250. NMDAR, NMDA receptor ; HK, hexokinase ; αCaMKII, α subunit of calcium/calmodulin-dependent protein kinase II. Molecular sizes in kilodaltons are shown at left.

of tyrosine phosphorylated proteins in the cerebellar PSD fraction with that of forebrain PSD, we carried out immunoblot analyses of the PSD fractions with phosphotyrosine-specific antibody (4G10). Fig. 2 shows that there are many more tyrosine-phosphorylated proteins in the cerebellar PSD than in the forebrain PSD. At the position of NMDA receptor subunit 2B (NR2B), which is the major tyrosine phosphorylated protein in the forebrain PSD[12], there did not appear a major immunoblot signal in the cerebellar PSD, indicating that either NR2B is not present in large amount or not tyrosine-phosphorylated as intensely as in forebrain. Interes-

tingly, a major phosphotyrosine signals was detected with a 50 kD molecular size (arrowhead). We named this protein as PSD-50. This position coincided with that of αCaMKII. However, the immunoblot signal in cerebellum was stronger than that of forebrain, while the band intensities of Coomassie stain were reverse. Thes results indicate that the responsible protein is different from αCaMKII.

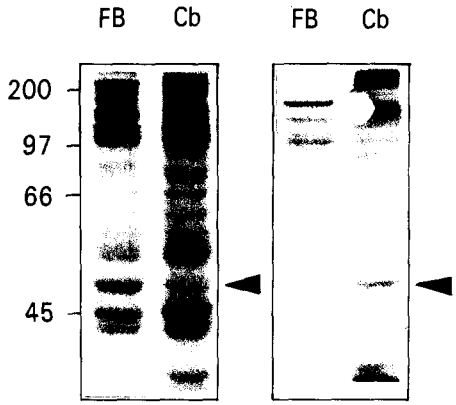

Fig. 3. Tyrosine-phosphorylated proteins in cerebellar and forebrain PSD fractions. Forebrain (FB) and cerebellar (Cb) PSD fractions (each 40 μg) were electrophoresed in 6% SDS-gels and either stained with Coomassie dye (left panel) or transferred to NC and immunoblotted with phosphotyrosine-specific antibody (right panel). A major tyrosine-containing protein (PSD-50) was indicated in both panels (arrowheads). Molecular sizes are shown in kilodaltons at left.

Association of PSD-50 with N-octylglucoside (NOG)-insoluble fraction

The PSD is composed of 'core' and 'associated' proteins. The core proteins are tightly aggregated each other and are not easily solubilized in a moderately harsh detergent such as Triton X-100 and NOG. To see if the PSD-50 is a core protein, the PSD fraction was treated with a final 1% NOG. The NOG-soluble (NOG-S) and -insoluble (NOG-P) fractions were separated in a 6% SDS-gel and immunoblotted with phosphotyrosine-specific antibody. Fig.4 shows that the PSD-50 is associated with the NOG-P fraction along with most other tyrosine phosphorylated proteins.

Fig. 4. Association of PSD-50 with NOG-insoluble fractions. PSD fractions were treated with 1% NOG and pellet (NOG-P) and supernatant (NOG-S) were separated by ultracentrifugation. Each 40 g and PSD fraction were electrophoresed and immunoblotted with a phosphotyrosine-specific antibody as in Fig. 3. A major phosphotyrosine-containing protein, PSD-50, was indicated as an arrowhead.

Discussion

In this report we have shown that 1) the overall SDS-PAGE profile of proteins in cerebellar PSD fractions is similar to that of forebrain PSD fractions, but differ in a few protein bands including about 50 kD band, 2) the major tyrosine-phosphorylated protein bands are different each other, 3) the major cerebellar tyrosine-phosphorylated protein band, PSD-50, comigrates with

αCaMKII, and 4) the PSD-50 is associated with NOG-insoluble fractions.

The PSD may have some molecules important in synaptic regulation[22]. Although cytoskeletal proteins such as actin, tubulin, and fodrins are known to constitute major components in PSD fractions[6], other molecules important in synaptic signal transduction were recently identified. Recently identified PSD proteins that may be important in synaptic regulations are 1) αCaMKII[7], the major PSD protein essential for induction of long-term potentiation of synaptic transmission[7-9], 2) PSD-95, a homolog of the Drosophila discs-large tumor suppressor protein[10-11], 3) the NMDA receptor subunit 2B (NR2B)[13], 4) densin-180, a new brain-specific synaptic protein of the O-sialoglycoprotein family[23]. Recently, PSD-95 has been shown to bind directly to the NMDA receptor subunit 2B (NR2B) through the second PDZ domain[24]. The NR2B is highly enriched and is the major tyrosine-containing protein of the forebrain PSD fraction [13]. However, although tyrosine phosphorylation is implied in receptor clustering and localization at specific synapses[22], little is known about other tyrosine-containing proteins in the PSD fraction.

Tyrosine phosphorylation has been shown to play major roles in the regulation of cellular growth, proliferation, and differentiation[14]. Tyrosine phosphorylation is also involved in the regulation of neuronal processes. Moreover, high concentrations of tyrosine kinases and their substrates at synapses suggest that tyrosine phosphorylation may regulate synaptic transmission[25]. The NMDA receptor, a subtype of glutamate receptors, is major Ca^{2+} channels in the postsynaptic membrane. After NMDA activation, calcium entry through NMDA receptors into the postsynaptic neuron is a critical event. Bading and Greenberg[17] reported that stimulation of cultured rat hippocampal cells resulted in the rapid and transient tyrosine phosphorylation of a 39-kD protein (p39), indicating tight regulation of tyrosine phosphorylation in synaptic regulation.

Moon et al.[13] reported that the NMDA receptor subunit 2B (NR2B), which is highly enriched in the PSD fraction, is the major tyrosine-containing protein in the forebrain PSD fraction. The tyrosine phosphorylation of NR2B, however, occurred at an early stage of neuronal development[26], suggesting that tyrosine phosphorylation of NR2B may be involved in neuronal development or synaptogenesis of certain synapses. Studies on other substrate proteins for tyrosine kinases are very limited. This report shows that tyrosine phosphorylation of proteins in cerebellar PSD fractions is significantly different from that of the forebrain PSD fraction. In overall profile, tyrosine-containing proteins in the cerebellum was more abundant in number, indicating that synaptic regulation between cerebellum and forebrain are quite different. Our result shows that the major tyrosine-containing protein in the cerebellar PSD fraction is a 50 kD protein, which we named PSD-50. In the forebrain PSD fraction, the major tyrosine-containing protein was the 180 kD NR2B[13]. The 180 kD region in cerebellar PSD lane of SDS-PAGE, however, did not show any major signal with phosphotyrosine-specific antibody, suggesting that the NR2B is not the major tyrosine-containing protein in the cerebellar PSD. In addition, the amount of NR2B in the cerebellar PSD fraction was minimal (unpublished result). The major phosphotyrosine protein band, PSD-50, in cerebellum comigrated with αCaMKII. However, the PSD-50 may not be CaMKII, because the signal strength with phosphotyrosine-specific antibody was stronger in cerebellum despite less amount of αCaMKII in cerebellum. The result strongly indicates that PSD-50 is different from CaMKII. Further studies are necessary to identify the PSD-50.

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초록 : 흰쥐 소뇌 연접후치밀질내 phosphotyrosine 함유 단백질에 대한 연구

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tyrosine 인산화효소계를 통한 신호전달은 신경의 발생과 연접활성조절에 중요한 역할을 한다. 흰쥐 소뇌의 연접후치밀질에 존재하는 tyrosine 함유 단백질들을 조사하기 위하여 immunoblot 분석을 한 결과, 소뇌 연접후치밀질의 전반적인 단백질조성은 전뇌와 비슷하였다. phosphotyrosine 특이성 항체로 immunoblot 한 결과 소뇌의 연접후치밀질에는 전뇌보다 많은 수의 단백질들이 tyrosine 인산화되어 있었으며, 전뇌와 달리 소뇌의 주된 tyrosine 인산화 단백질은 50 kD 크기의 새로운 단백질이었다. PSD-50로 명명한 이 단백질은 SDS-PAGE에서 αCaMKII와 같은 위치에 이동하였다. 그러나 소뇌에는 전뇌에 비하여 적은 량의 αCaMKII가 존재함에도 불구하고 전뇌보다 더 강한 phosphotyrosine immunoblot signal을 보이는 것으로 보아 PSD-50는 아마도 αCaMKII와는 다른 단백질로 추정된다.