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Differential Subsynaptic Distribution of BiP, ERp72 and Calnexin 88 in the Rat CNS Synapse

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ABSTRACT

Long-lasting changes in synaptic efficacy require newly synthesized proteins being deposited at the synapses. Recent evidence indicates that protein synthesis can occur at or near synapses. In this study, we searched for molecular chaperones in the synapse by immunoblot analysis. BiP and calnexin 88 were present in both forebrain and cerebellar PSD fractions. However, ERp72 was associated only with forebrain PSD fraction. BiP and ERp72 were enriched by ~2- and 7-fold in the synaptosome fraction compared to brain homogenates but weakly associated with the PSD. In contrast, calnexin 88 was enriched by ~2- and ~2.5-fold in the synaptosome and PSD fractions, respectively, compared to homogenates, indicating that calnexin is associated with the PSD. Our results indicate that chaperones are differentially distributed in the synapse, and that protein folding machinery is present at or near synapses.

Key words: BiP, calnexin, chaperone, ERp72, synapse, immunoblot, PSD

INTRODUCTION

By now, it is well accepted that synapses, the points of contact between neurons, are dynamic and display a remarkable experience-dependent plasticity. This plasticity accompanies both morphological and biochemical changes and underlies long-term memory storage (Segal and Andersen, 2000). A neuron in the mammalian central nervous system (CNS) has many thousands of synaptic inputs. However, the plasticity must occur at specific synapses that receive synaptic input. Although short-lived changes in synaptic strength are probably the result of modifications of existing proteins at synapses, long-lasting changes require participation of new proteins. Although some of these proteins are derived from newly transcribed genes, recent findings suggest that they are synthesized at or near involved synapses. In theory, the synthesis of proteins at or near synapses provides a mechanism by which synapses can independently control their strength, circumventing the need for precisely ad-

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dressed protein transport from the soma (Schuman, 1999).

The first evidence for local dendritic protein synthesis is provided by morphological observation. Steward and Levy (1982) discovered preferential localization of polyribosomes under the base of dendritic spines in granule cells of the dentate gyrus. In addition to ribosomes, neuronal dendrites are equipped with various components for translation such as mRNA, tRNA, initiation and elongation factors (reviewed in Steward and Schuman, 2001). Furthermore, biochemical evidencegested that glycosylation occurs within dendrites (Torre and Steward, 1996), indicating that essential elements required for protein synthesis and posttranslational modifications are present at the base of spines.

A prominent feature of the excitatory synapse is the postsynaptic density (PSD), a cytoskeletal specialization that is attached to the postsynaptic membrane. The PSD contains receptors for neurotransmitters, receptor-associated proteins and scaffold proteins. Therefore, the PSD serves as a general signaling machine that links neurotransmitter receptors to downstream effector molecules (Kennedy, 2000). Within the PSD may lie clues to the mechanisms of the most intriguing of brain functions, including activity-dependent changes in synaptic strengthening. Recently, we have found that heat shock proteins 70 (HSP70; Hsc70 and Hsp70) are associated with rat cortical and hippocampal synapses (Moon et al., 2001). Since the heat shock proteins (HSPs) serve as molecular chaperones in protein holding and folding, presence of HSP70 in the PSD strongly indicates that newly synthesized proteins can be 'kidnapped' and folded at the synapse and could provide a potential candidate system for a 'synaptic tag'. In this study we further investigated if other chaperones are present in the synapse, and report that BiP, ERp72, and calnexin are differentially distributed in the synapse.

MATERIALS AND METHODS

Antibodies

Monoclonal antibodies against pan-actin and $GABA_A$ α1 subunit were purchased from Boehringer Mannheim and PharMingen (San Diego, USA), respectively. Monoclonal and polyclonal antibodies were used at 1 : 2,000 and 1 : 5,000 dilution, respectively.

Subcellular fractionation

The One-Triton PSD fraction (Cho et al., 1992) was prepared from adult rat (Sprague-Dawley, ~200 g) forebrains by washing synaptosome-enriched fraction with 0.5% Triton X-100 as described previously (Carlin et al., 1980; Cho et al., 1992).

Immunoblot

After SDS-PAGE, proteins were transferred to nitrocellulose (NC) membrane which was blocked overnight at 4^oC with TTBS [0.2% Tween-20, 10 mM Tris-HCl (pH 7.5), and 0.2 M NaCl]. Blots were incubated with primary antibodies [mouse anti-actin monoclonal (1 : 2,000), anti-$GABA_A$ α1 (1 : 2,000); rabbit polyclonal anti-synapsin, -ERp72, -BiP, -calnexin (1 : 5,000)] for 2 h at room temperature. Blots were rinsed in TTBS for 4 times (20 min each) and incubated with alkaline-phosphatase-conjugated secondary antibodies (Boehringer Mannheim) for 2 h. Blots were developed according to the supplier's instructions.

RESULT AND DISCUSSION

We first investigated association of chaperones with synapses from forebrain (FB) and cerebellum (CBL). PSD fractions were isolated from each brain region and the proteins were separated in SDS-PAGE. Coomassie-stain of SDS-gels showed similar overall protein band profiles between two fractions (Fig. 1A, Coom.). The integrity of each fraction was tested by immunoblotting with two antibodies. The first antibody we used was anti-synapsin. The synapsin is a synaptic vesicle protein that is present in both excitatory and inhibitory synaptic terminals. Therefore, this protein is expected to be present in similar amounts in the two fractions. As expected, the immunoblot signal intensities were comparable between the two fractions (Fig. 1A, α-syn). The second antibody employed is α-$GABA_A$ α1, a subunit of GABA receptor. Since cerebellum is abundant in inhibitory synapses, it is expected that GABA receptors are present in higher density in the cerebellar PSD than in FB PSD. As expected the immunoblot signal in cerebellar PSD lane is

much stronger than that in the FB PSD (Fig. 1A, α-GABA). Taken together, these results indicate that the isolated PSD fractions are integral and specific to each brain regions.

In order to address subcellular distribution of a protein, it is essential to compare the amount of the protein in equal amounts of the total fraction. To verify equal amount of proteins in each fraction, we used a monoclonal antibody against pan-actin, because actin is expected to be present in more or less equal density throughout plasma membranes. Forebrain homogenate, synaptosome, and PSD fractions were assayed for protein contents. When each fraction was electrophoresed in a SDS-gel and stained with Coomassie, the stain intensities of most protein bands are similar and the bands are integral with little degradation (Fig. 1B, Coom.). When a sister SDS-gel was transferred to nitrocellulose and blotted with α-pan-actin antibody, it revealed very similar immunoblot signals among the lanes. These results indicate that the subcellular fractions are integral in terms of protein quality and the amount of proteins are more or less equal. Using these subcellular fractions, we first investigated association of chaperones with synapses from different brain regions. When FB and CBL PSD fractions were subjected to immunoblot analysis, BiP and calnexin 88 were shown to be present in comparable amounts in the two fractions (Fig. 2, α-BiP and α-calnexin). However, ERp72 was highly associated with FB PSD but minimally with CBL PSD (Fig. 2, α-ERp72). These results suggest that chaperones are differentially associated with PSD fractions depending on brain regions.

The PSD is an organelle rich in hydrophobic and filamentous proteins. The PSD is therefore 'sticky', a feature that causes contamination of non-PSD proteins being included in the 'PSD fraction' during isolation. Therefore, it is important to compare subsynaptic distribution of a protein; if a certain

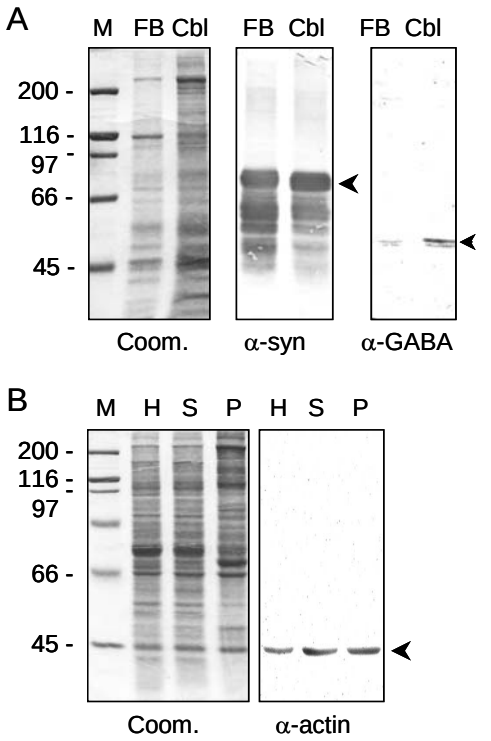

Fig. 1. Integrity of subcellular fractionation. **A,** PSD fractions. PSD fractions from rat forebrain (FB) and cerebellum (Cbl) were isolated and 40μg of each fraction were electrophoresed in a 8% SDS-gel. Gels were either stained with Coomassie R-250 (coom.) or transferred to nitrocellulose membrane and blotted with α-synapsin (syn) or α-$GABA_A$ α1 antibody (1 : 2,000). **B,** Subcellular fractions. Homogenate (H), synaptosome (S) and PSD (P) fractions were isolated from rat forebrain, and 40μg of each fraction were electrophoresed in a 6% SDS-gel. The gel was either stained with Coomassie R-250 (Coom.) or transferred to nitrocellulose membrane and blotted with α-actin antibody (α-actin, 1 : 2,000). Positions of relevant signals were marked by arrowheads. Molecular sizes are shown at left in kilodaltons (kDa).

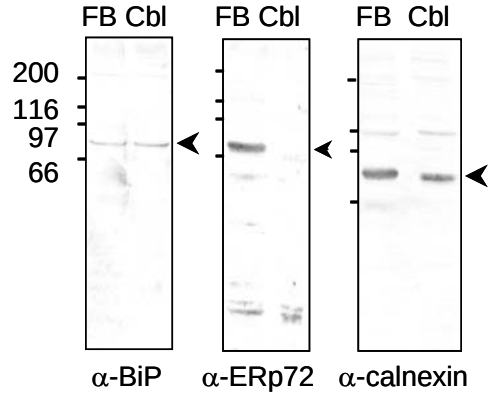

Fig. 2. Regional difference in the association of chaperones with PSD fractions. Forty micrograms of forebrain (FB) and cerebellar (Cbl) PSD fractions were electrophoresed in a 10% SDS-gel, transferred to nitrocellulose and blotted with indicated polyclonal antibodies (1 : 5,000). Positions of relevant signals were marked by arrowheads. Molecular sizes are shown at left in kilodaltons (kDa).

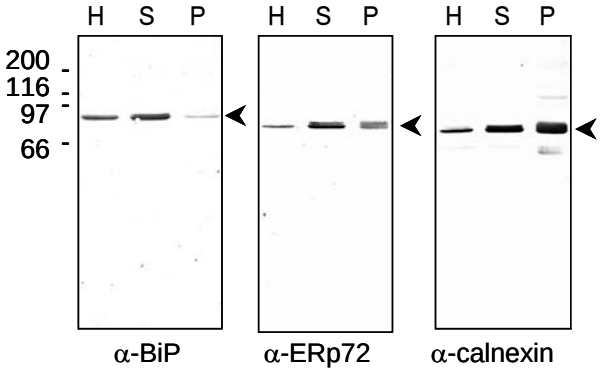

Fig. 3. Differential subsynaptic distribution of chaperones in the forebrain PSD fraction. Homogenate (H), synaptosome (S) and PSD (P) fractions were isolated from rat forebrain, and 40μg of each fraction were electrophoresed in a 10% SDS-gel, transferred to nitrocellulose membrane and blotted with indicated polyclonal antibodies (1 : 5,000). Positions of relevant signals were marked by arrowheads. Molecular sizes are shown at left in kilodaltons (kDa).

protein is a member of the PSD, it will be enriched in the PSD fraction when compared to brain homogenate or synaptosome fractions. We investigated subcellular distribution of chaperones in the forebrain. Immunoblot analysis revealed that BiP and ERp72 were enriched by ~2- and 7-fold, respectively, in the synaptosome fraction (Fig. 3, α-BiP and α-ERp72), indicating that these chaperones are present in high concentration in the synaptic region. However, these chaperones were weakly associated with the PSD (Fig. 3, α-BiP and α-ERp72), indicating that they are not associated with the PSD. In contrast, calnexin 88 was enriched by ~2- and ~2.5-fold in the synaptosome and PSD fractions, respectively, compared to homogenates (Fig. 3, α-calnexin), indicating that calnexin is associated with the PSD.

BiP, ERp72 and calnexin 88 are known to be molecular chaperones in the endoplasmic reticulum (ER). Our results indicate that BiP and ERp72 are not associated with the PSD but highly concentrated in the synaptic region. It is reported that BiP- and calnex-positive cisternae are present in close vicinity to synapses (Wenthold, 1999). Therefore, it is highly possible that these proteins, which are present in the ER cistenae at the base of synapses, are included in the synaptosome fraction during isolation. BiP is a member of the highly conserved HSP 70 family of proteins and is constitutively expressed in the ER (Munro and Pelham, 1986; Wang et al., 1998), and binds unfolded regions on proteins containing hydrophobic residues (Flynn et al., 1991; Blond-Elguindi et al., 1993). ERp72 is a member of the thioredoxin family of proteins, and forms intermolecular disulfide-bonded complexes (Reddy and Corley, 1998). Such protein folding and disulfide formation may occur in ER-like cisternae at subsynaptic regions and provide nearby synapses with new proteins during synaptic plasticity.

In contrast to BiP and ERp72, calnexin was highly enriched in both synaptosome and PSD fractions, suggesting that it is associated with the PSD. Calnexin is known to be a major ER protein and interact with nascent glycoproteins (Tatu and Helenius, 1997; Zhang et al., 1997). Cytosolic proteins, which a PSD protein would interact, are not glycosylated. A possible interpretation may be that calnex is present in cisternae that are present in the spine head. It is known that a specialized membrane structure, called raft, is present in the dendritic spine and sometimes in contact with the PSD (Suzuki, 2002). Therefore, it is highly possible that the calnexin present in this dendritic raft is isolated in the PSD fraction. In sum, our results indicate that protein folding machinery is present at or near synapses. Further studies such as electron microscopic observation are necessary to understand exact localization of these proteins in the synapse.

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