Chapter 2

Co-immunoprecipitation Methods to Identify Associated Proteins with Estrogen Receptor α at Postsynaptic Density in Brain Tissue

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Abstract

Co-immunoprecipitation is a traditional method but still one of the most powerful tools to identify novel interaction partners by increasing its specificity and decreasing false-positive result. In the current work, we show a robust procedure where the use of two sequential approaches (separation of postsynaptic density by centrifuge separation method in combination with detergent treatment and the use of specific antibodies against target proteins for its precipitation) improved the specificity of co-immunoprecipitation methods. Mass spectrometric analysis also helped to identify novel-associated proteins with target proteins without any bias of suspected interaction partners.

Key words Co-immunoprecipitation, Centrifuge separation, Brain, Estrogen receptor, Postsynaptic density, Chromatin immunoprecipitation, Major histocompatibility complex

1 Introduction

1.1 Co-immunoprecipitation: A Powerful Tool for Protein-Protein Interactions

Information of protein-protein interactions is very important to investigate the molecular mechanisms which underlie various physiological events. To identify associated proteins with targeted proteins, several novel methods have been reported, and some traditional methods have been improved over decades [1]. Among these methods, co-immunoprecipitation, one of the most traditional methods, is still one of the most powerful tools and the most often used for this purpose [2]. This is probably due to its high sensitivity and relatively reliable detection of associated proteins using specific antibodies under native conditions without overexpressions of target proteins and compatibility with mass spectrometric identification of proteins. Chromatin immunoprecipitation (ChIP) is also a method of co-immunoprecipitation to identify associated DNA fragments with target proteins and offers high sensitivity and reliable detection of associated DNA fragments

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using polymerase chain reaction. This method has also given us a lot of information about mechanisms underlying physiological events [3].

1.2 Limitation of Co-immunoprecipitation

On the other hand, co-immunoprecipitation has some limitations. One of the limitations is that this method cannot distinguish direct and indirect interactions. Thus, identified associated proteins by this method can interact directly with target proteins or indirectly with target proteins as one of the proteins in the complex including the target proteins. Another difficulty is that this method often leads to false-positive results, although this possibility is considered to be much less than other methods such as yeast two-hybrid method [2]. This problem is mainly originated from non-specific binding of antibodies which is used for precipitation of target proteins. This tendency is much more evident when using tissue samples than cell culture samples. This is probably because tissue samples include various types of cells that do not express target proteins. Particularly in brain tissues, only 10% of cells are neurons, and the other 90% of cells are glial cells. Therefore, the portion of target proteins particularly expressed in specific neurons is considered to be much smaller. This false-positive result occurs more often when target proteins are expressed less than other proteins despite its important roles in physiological events. As we will see later, estrogen receptor alpha (ERα), famous for regulating reproductive functions in reproductive organs, is a good example because expression level of ERα in the hippocampus is lower than one tenth of ERα expressed in the ovary [4]. However, decades of studies have demonstrated that ERα in the hippocampus plays important roles in the regulation of synaptic plasticity [4–6].

1.3 Improving Specificity of Co-immunoprecipitation

An approach to overcome these problems is using antibodies which show good specificity for the target proteins. Although antibodies predominantly bind to the epitope within target proteins, other non-specific proteins without the epitope can also interact with the antibodies. Specificity of antibodies is decided by the ratio of bindings to specific proteins to non-specific proteins, and antibodies with low specificity tend to lead to false-positive results. This specificity also depends on the type of antibodies such as polyclonal or monoclonal. Particularly, polyclonal antibodies tend to have large variability of their specificities, and purification of antibodies is particularly important for improving their specificities. Polyclonal antibodies with different lot numbers may also have different specificities. Therefore, it is important to evaluate the specificity of antibodies before co-immunoprecipitation, particularly when using brain tissues.

Another approach to overcome the problems is separation of samples before co-immunoprecipitation. This pre-separation step can easily remove non-specific proteins and increase the ratio of

antibody bindings to specific proteins to non-specific proteins in the co-immunoprecipitation experiment. Centrifuge separation is suitable for the step of pre-separation because most of proteins are localized at a specific subcellular region such as the nucleus, cellular membrane, endoplasmic reticulum, and cytoplasm. Centrifuge separation can easily separate these specific regions from others and remove a large part of nontarget proteins. Furthermore, centrifuge separation in combination with detergent treatment can separate neuron-specific subregions such as the postsynaptic density (PSD) or presynaptic vesicles which play important roles in the regulation of synaptic transmissions and plasticity in the brain [7–9]. Some proteins are localized at several subcellular regions at the same time by associating with different proteins, having different roles in physiological events. Centrifuge separation of a specific subcellular region allows us to investigate target proteins in a particular region by removing the other regions. Therefore, co-immunoprecipitation with specific antibodies following centrifuge separation makes possible to easily and reliably detect associated proteins with target proteins localized at a specific subcellular region.

After co-immunoprecipitation, associated proteins are generally detected by western blotting using specific antibodies against suspected interaction partners with the target proteins. Although antibodies detect associated proteins with high sensitivity, this property may also lead to false-positive results of detecting a small amount of contaminated proteins. To reduce this possibility, it is general to reverse co-immunoprecipitation where antibodies against detected interaction partners are used for co-immunoprecipitation and antibodies against original target proteins are used for the detection in western blotting. However, western blotting detection cannot discover novel interaction partners. Mass spectrometric analysis is the most powerful method to identify associated proteins with high sensitivity without any bias of suspected interaction partners.

In this chapter, we show how these approaches improve the specificity of co-immunoprecipitation by introducing a study that identified associated proteins with estrogen receptor α (ERα) localized at the PSD in rat brain. ERα is a nuclear receptor and localized in the cytoplasm in the absence of its ligand, 17β-estradiol. By binding with estradiol, ERα is transferred into nuclei and binds with estrogen response element to regulate gene expressions under the element [10]. In addition to this slow/genomic (within days) functions, it has been found that ERα is also localized at the PSD, and its binding with estradiol rapidly (within 2 h) modulates synaptic plasticity [4–6]. Although it is considered that ERα is localized at the PSD by anchoring to its scaffold proteins, these associated proteins have not been identified. Using co-immunoprecipitation with specific antibodies and centrifuge separation method in combination with a detergent treatment, we

successfully purified ERα localized at the PSD and identified its associated proteins by mass spectrometry.

2 Materials and Methods

2.1 Evaluation of Antibody Specificity Against Estrogen Receptor α

Before co-immunoprecipitation, our group assessed specificity of six antibodies against ERα by western blotting using the rat hippocampus as a brain tissue sample. The ovary was also used as a positive control. These antibodies included homemade polyclonal antibody RC-19 which was column-purified with antigens of rat and mouse ERα C-terminal 19 amino acids (C-HSLQTYYIPPEAEGFPNTI) [4]. Other antibodies were commonly used or commercially available antibodies. MC-20 is polyclonal antiserum against C-terminal 20 amino acids of rat and mouse ERα [11]. Using RC-19, we observed only a single protein band at 67 kDa of ERα molecular weight in the hippocampus homogenate and a very strong band at the same molecular weight in the ovary homogenate (Fig. 1a). We also confirmed that this 67 kDa band was absent in the hippocampus of ERα knockout mice [4, 5]. On the other hand, MC-20 is one of the most often used antiserums to detect ERα and showed a band at 67 kDa in the ovary homogenate. However, this 67 kDa band was not detectable in the hippocampus sample, and major bands were observed at different molecular weights. MC-20 also showed these non-specific bands in the hippocampus of ERα knockout mice [4]. This tendency also can be observed in immunohistochemistry [4]. The staining of RC-19 can be observed in the hippocampus of wild-type mice but not in ERα knockout mice. On the other hand, MC-20 staining was observed in both genotypes. These results show that the use of antibodies purified with antigens from antiserum is important to increase the specificity, particularly in tissues where target protein expression is low. Therefore, RC-19 is expected to have high specificity for ERα in the co-immunoprecipitation experiment. It is important to know that the specificity of antibodies cannot be necessarily the same between different experiments such as immunoprecipitation, western blotting, and immunohistochemistry. However, evaluation of antibody specificity in an experiment can still give important information of the specificity in another experiment.

2.2 Centrifuge Separation of Postsynaptic Density

There are various choices of methods which can be used for purification of proteins before co-immunoprecipitation. Centrifuge separation is a traditional method to separate subcellular regions and suitable for co-immunoprecipitation. In general centrifuge separation protocol, tissue samples can be separated to the nuclear fraction (P_1), plasma membrane fraction (P_2), microsomal fraction (P_3), and cytoplasmic fraction (S_3). In brain tissues, the P_2 fraction

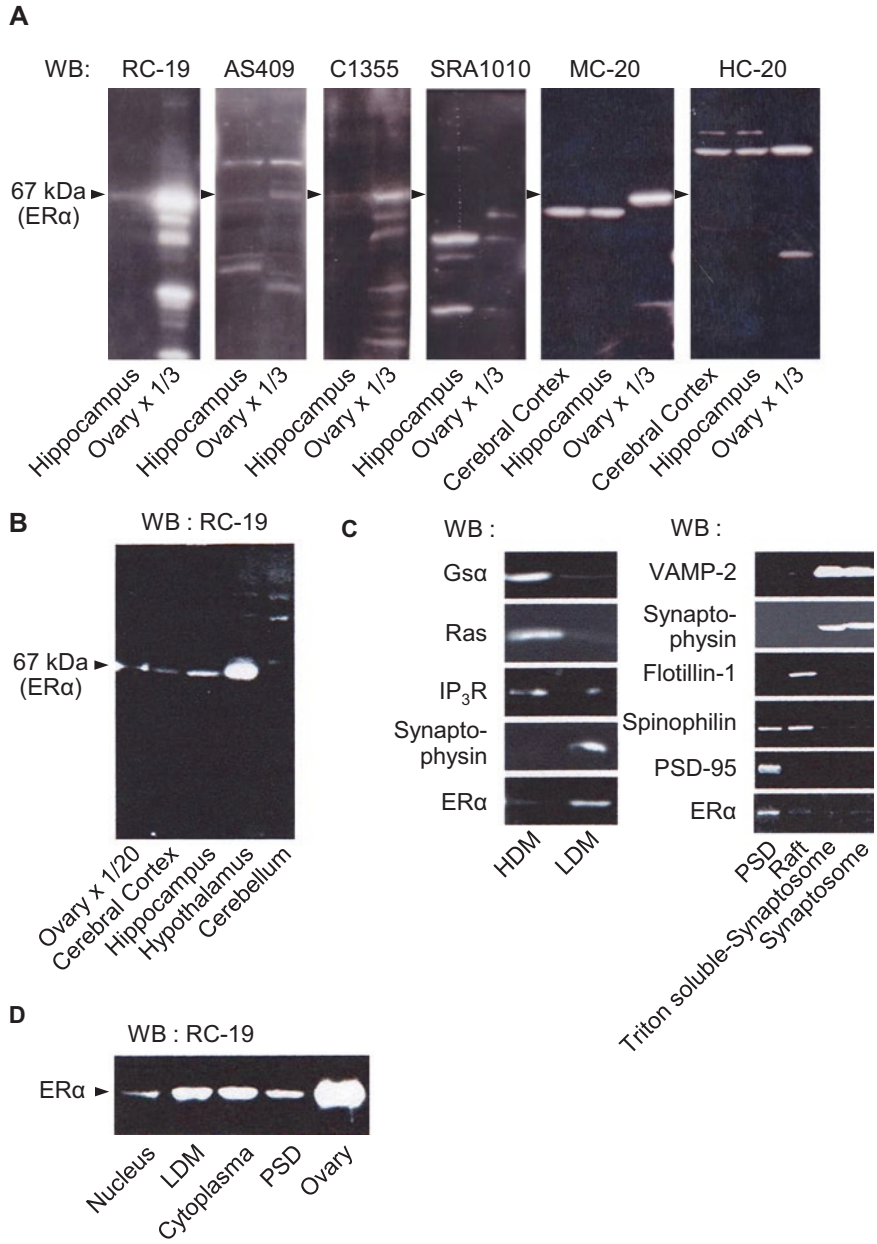

Fig. 1 Western blotting analysis using rat brains. (**a**) Western blotting of ERα with six antibodies to evaluate their specificities. The hippocampus and ovaries diluted to one third of hippocampal proteins were used as a brain sample and positive control, respectively. (**b**) Western blotting of ERα with RC-19 in some brain regions such as the cerebral cortex, hippocampus, hypothalamus, and cerebellum. The ovary sample diluted to 1/20 of proteins in other brain regions was also used as a positive control. (**c**) Western blotting of several region-specific markers in fractions prepared by centrifuge separation such as high-density membrane (HDM), low-density membrane (LDM), postsynaptic density (PSD), raft, triton-soluble synaptosome, and synaptosome. (**d**) Western blotting of ERα with RC-19 in fractions prepared by centrifuge separation such as the nucleus, LDM, cytoplasm, and PSD. The ovary sample was also used as a positive control

can be further separated to the myelin, synaptosomal, and mitochondrial fractions [9]. The postsynaptic density (PSD) fraction can be separated from the synaptosomal fraction by centrifuge separation in combination with detergent treatment [7, 8].

Our group has applied this method to separate ERα localized at the PSD (Fig. 2a). Two male Wistar rats aged 12 weeks (Saitama Experimental Animal Supply, Saitama, Japan) were deeply anesthetized and decapitated. Then, the whole brains were quickly removed and placed in ice-cold homogenization buffer, containing 0.32 M sucrose, 5 mM HEPES, 25 mM KCl, 4 mM EDTA-4Na, 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitor cocktails (Roche, Laval, QC, Canada), and pH was adjusted to 7.3. Because synaptosomes are caused to coacervate by high concentrations of univalent ions or low concentrations of divalent ions, it is important to optimize the concentration of these ions in the homogenization buffer [9]. We used whole brains because the localization of ERα at the PSD and its rapid modulation of synaptic plasticity have been shown in various brain regions. However, we removed cerebellums because this region contains much less ERα than other brain regions (Fig. 1b). The brain sample was homogenized (10% wt/vol) in homogenization buffer and centrifuged at 830 × *g* for 10 min. The pellet was used as P_1, although this fraction contains cell debris in this protocol. Supernatant from the first centrifugation was subjected to centrifugation at 10,000 × *g* for 20 min, and the pellet (P_2) was retrieved.

The P_2 fraction contains the synaptosomal fraction, which consists of synaptic terminals pinched-off from neurons by homogenization, and pre- and postsynapses are included in this fraction [9]. The synaptosomal fraction is generally prepared by subsequent ultracentrifuge separation of the P_2 fraction using 0.85/1.0/1.2 M sucrose step gradient and collected from the interface between 1.0 M and 1.2 M sucrose. Although relatively pure PSD fraction is separated from this fraction (Fig. 1c), we skipped the step of synaptosomal separation to avoid loss of target proteins. The PSD fraction is separated by its characteristic biochemical properties of detergent resistance [7, 8]. Because the PSD consists of tightly packed proteins important for synaptic transmissions such as receptors for neurotransmitters, cell adhesion proteins, scaffold proteins, and signaling molecules [12], the PSD complexes cannot be dissociated by mild detergents such as Triton X-100. Thus, P_2 was solubilized in 40 mL homogenization buffer without sucrose containing 0.5% Triton X-100 for 30 min at 4 °C using a stirrer, and the triton-insoluble P_2 pellet fraction was collected by centrifugation at 15,000 × *g* for 30 min and used as PSD-rich samples for co-immunoprecipitation. Further centrifugation of the supernatant obtained from the fractionation of P_2 at 25,000 × *g* for 60 min gave the high-density membrane fraction. The resultant supernatant was centrifuged again at 100,000 × *g* for 60 min, and the

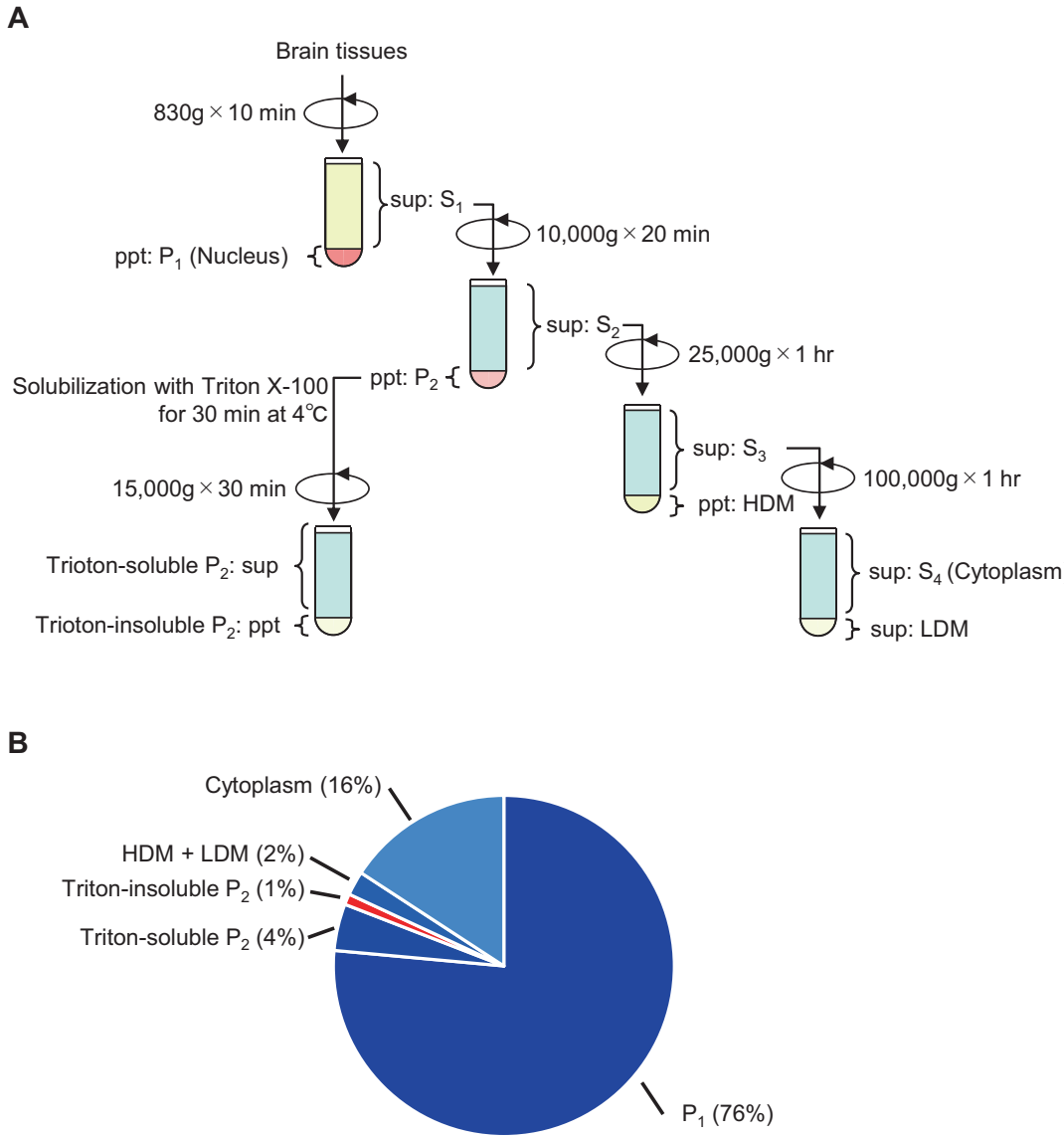

Fig. 2 Centrifuge separation of triton-insoluble P_2 fraction. (**a**) Schematic diagram of centrifuge separation. (**b**) Proportion of protein amounts in fractions prepared by centrifuge separation. P_1 (nucleus), triton-soluble P_2, triton-insoluble P_2, P_3 (membrane), and S_3 (cytoplasm) account for 76%, 4%, 1%, 2%, and 16% of total proteins, respectively

pellet and the supernatant were collected separately as the low-density synaptic membrane fraction (LDM) and cytoplasm fraction, respectively [4]. The LDM faction contained proteins specifically localized at synaptic vesicles (Fig. 1c). Western blotting analysis showed that ERα was localized at the PSD fraction as well as nucleus, cytoplasm, and LDM fractions (Fig. 1d). This centrifuge separation removed ERα in subregions other than the PSD and allows us to analyze the associated proteins with PSD-localized

ERα. It is important that, the triton-insoluble P_2 fraction contained less than 1% of total proteins in the brain homogenate (Fig. 2b). Therefore, this single step of centrifuge separation greatly reduced nontarget proteins and is expected to increase the specificity of co-immunoprecipitation more than 100-folds.

2.3 Co-immunoprecipitation of Estrogen Receptor α-Associated Proteins

Although solubilization of samples is necessary before co-immunoprecipitation, the use of strong detergents or high concentration of detergents interferes protein-protein interactions. Thus, it is important to optimize the combination of detergents and their concentrations to solubilize samples but not to interfere the association of target proteins. As we used in this experiment, low concentration of sodium dodecylsulfate (SDS) combined with mild detergents of sodium deoxycholate and NP-40 is often used for solubilization of the PSD [7, 8]. We solubilized triton-insoluble P_2 pellet using a stirrer for 1 h at 4 °C in 3 mL immunoprecipitation buffer containing 0.1% SDS, 1% sodium deoxycholate, 1% NP-40, 5 mM HEPES, 25 mM KCl, 4 mM EDTA-4Na, 1 mM Na_3VO_4, 50 mM NaF, and protease inhibitor cocktails (Roche), and pH was adjusted to 8.1.

For precipitation of target proteins, we need to select beads to link with antibodies, such as agarose, sepharose, or magnetic beads linked to protein G, protein A, or secondary antibodies. Our group compared these beads and selected magnetic beads linked to anti-rabbit IgG (Dynabeads M-280 anti-rabbit IgG; Thermo Fisher Scientific Inc., Waltham, MA, USA) for the following reasons. First, some of these beads non-selectively associate with proteins in samples and possibly lead to false-positive results. Anti-rabbit IgG-linked magnetic beads showed less associations with non-specific proteins than other beads. Second, centrifugation is necessary to collect agarose and sepharose beads in co-immunoprecipitation. However, centrifugation also non-specifically precipitates insoluble protein complexes. Precipitation of insoluble protein complexes was also observed even when these insoluble complexes were removed by several centrifugations of triton-insoluble P_2 samples before co-immunoprecipitation. The use of magnetic beads solved this problem because these beads can be collected on the wall of sample tubes by a magnet attached to the outside of the wall without centrifugation.

Our group reacted 6.7×10^7 of magnetic beads linked to anti-rabbit IgG with 10.0 μg of RC-19, a homemade rabbit polyclonal antibody against ERα, in phosphate buffer saline containing 0.1% bovine serum albumin by rotating sample tubes for 3 h at 4 °C. Then, RC-19 was covalently coupled with anti-rabbit IgG by dimethyl pimelimidate (Pierce, Rockford, IL, USA) to minimize the contamination of RC-19 in the elution process of target proteins. After washing with immunoprecipitation buffer, the magnetic beads linked with RC-19 were reacted with 1.5 mL

triton-insoluble P_2 sample by rotating sample tubes overnight at 4 °C. It is important to prepare a negative control using normal rabbit serum (NRS) exactly the same as RC-19 for the identification of non-specifically associated proteins. For separation of SDS-PAGE, ERα was eluted by sample buffer containing 125 mM Tris-HCl (pH 6.8), 5 mM 2-mercaptoethanol, 10% sucrose, 6% sodium dodecylsulfate (SDS), and 0.002% bromophenol blue by incubating for 5 min at 100 °C, followed by further elution by adding dithiothreitol (100 mM) and incubating for 10 min at 100 °C. After protein separation by SDS-PAGE, these gels were stained overnight with 0.1% silver using the silver stain MS kit (Wako, Tokyo, Japan).

In the silver staining analysis, many protein bands were observed in precipitated samples not only with RC-19 but also NRS at the same molecular weight (Fig. 3). This result shows that many proteins were non-specifically precipitated with antibody-linked magnetic beads, while we increased specificity of co-immunoprecipitation in various steps. However, some specific bands were observed particularly in RC-19 but not in NRS samples, and these bands were considered ERα-associated proteins. We also precipitated and silver-stained ERα-associated proteins in the LDM fraction. We can see that the patterns of specific bands precipitated with RC-19 were quite different between the triton-insoluble P_2 and LDM samples. This difference indicates that ERα is localized in the different subcellular regions associating with different proteins.

2.4 Mass Spectrometric Analysis of ERα-Associated Proteins

For mass spectrometric identification of ERα-associated proteins, RC-19-specific bands were isolated from the gels using razor blades. These gel fragments were destained with the destaining solution of the silver stain kit, and the proteins were digested in-gel with trypsin (V5280; Promega, Madison, WI, USA). Digested peptides were extracted from gel pieces with 50 μL 50% acetonitrile and 5% trifluoroacetic acid. ZipTip (Millipore, Billerica, MA, USA) was used for purification of peptides. A matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometer (Shimazu, Tokyo, Japan) was used for detection of ERα-associated proteins. Samples were prepared by mixing peptide solution with 0.3 μL matrix solution on a MALDI plate. All masses are reported as monoisotopic mass values. Peptides were identified using the Mascot search program (http://www.matrixscience.com) to perform theoretical trypsin digests and search for potential unmodified tryptic peptides.

Many tryptic peptides prepared from gel fragments of 67 kDa in the triton-insoluble P_2 and LDM samples were attributed to the tryptic peptides from ERα the same as our previous report [4]. We also identified precipitation of ERα by western blotting using RC-19 (Fig. 3b). When antibodies raised from the same animal

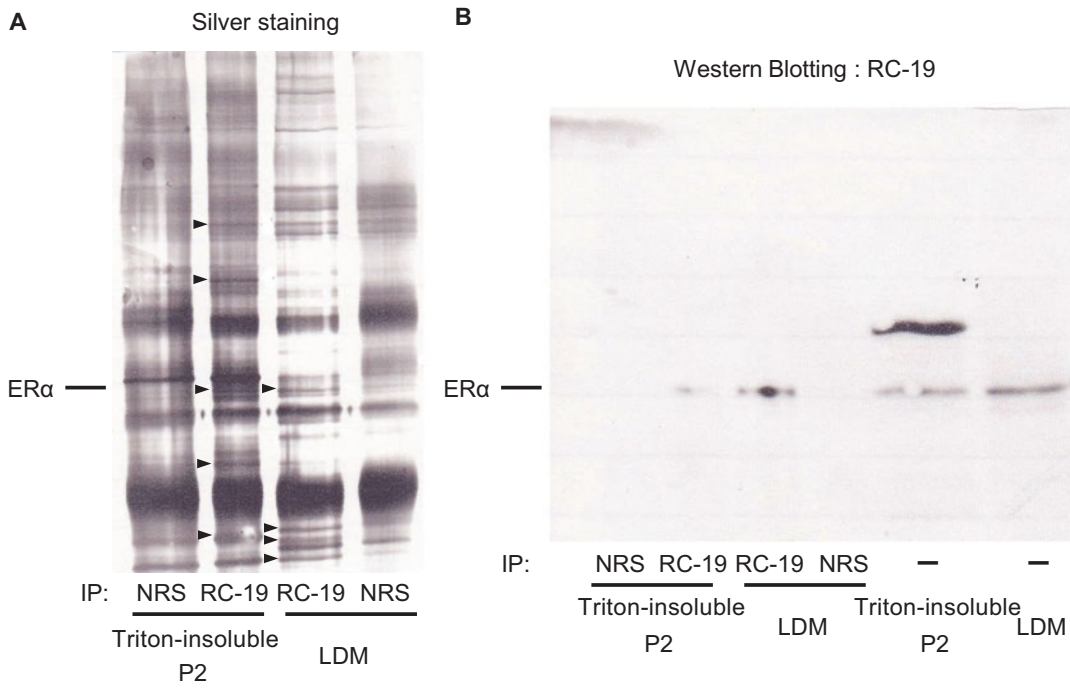

Fig. 3 Analysis of co-immunoprecipitated proteins. (**a**) Silver staining analysis of co-immunoprecipitated proteins by RC-19 and normal rabbit serum (NRS) in the triton-insoluble P_2 and LDM fractions. Non-specific bands were observed in precipitated samples with RC-19 and NRS at the same molecular weight. Some specific bands were also observed in RC-19 but not in NRS samples as indicated by arrowheads. (**b**) Western blotting analysis of ERα by RC-19 in original and co-immunoprecipitated samples prepared from the triton-insoluble P_2 and LDM samples. ERα was identified in all samples

species were used in western blotting and co-immunoprecipitation, it is convenient to detect proteins using secondary antibodies against non-denatured primary antibodies (Easy Blot; GeneTex, Irvine, CA, USA) in western blotting. This is because the covalently linkage of primary antibodies with beads cannot completely exclude contamination of the primary antibodies in the process of elution. However, secondary antibodies against non-denatured IgG detect only primary antibodies used in western blotting but not in co-immunoprecipitation.

Although other RC-19-specific bands were possibly attributed to ERα-associated proteins, most of them were unnamed proteins. In these proteins of the triton-insoluble P_2 sample, one of the most well-known and intriguing proteins was major histocompatibility complex (MHC) class II. MHC is an important immune protein and divided into three main subgroups, class I, II, and III. Although MHC class I is expressed in nearly all nucleated cells, including neural cells, the brain has traditionally been considered an immune-privileged region. Recently, it has gained much atten-

tion that MHC class I is expressed at postsynapses and suppresses glutamatergic synaptic transmissions [13–15]. MHC class II is also involved in the pathogenesis of various neural diseases [16, 17], while its roles in healthy brain have not been investigated. Therefore, it is interesting that ERα is associated with MHC class II at the PSD of neural cells. Although the role of this interaction in the brain will be investigated in future studies, this information shows a possibility that both proteins are involved in the same complexes which regulate synaptic plasticity at the PSD.

3 Conclusion

Although co-immunoprecipitation is a traditional method, this method is still one of the most powerful tools to identify novel interaction partners by increasing its specificity and decreasing false-positive result. These improvement procedures for the specificity of co-immunoprecipitation can be used not only for the analysis of protein-protein interactions but also protein-DNA interactions in ChIP experiments [3]. As we observed in the detection of ERα-associated proteins, the use of specific antibodies against ERα for its precipitation improved specificity of co-immunoprecipitation. PSD separation by centrifuge separation method in combination with detergent treatment also decreased non-specific proteins to less than 1% and increased the specificity of co-immunoprecipitation more than 100-folds. Finally, mass spectrometric analysis successfully identified a novel ERα-associated protein, MHC class II, without any bias of suspected interaction partners.

In decades, various biological methods and their sensitivities have been much improved [1]. This improvement makes possible to analyze proteins expressed at very low levels, while they play important roles in physiological events. We showed that proteins in the PSD are less than 1% of total proteins in the brain. Their expressions in this microstructure are very low despite their importance in synaptic transmissions and plasticity. ERα and MHC are also expressed at very low levels in the brain although both proteins play important neuron-specific roles. Our group successfully identified an interaction of ERα with MHC class II at the PSD of neural cells. In future, many low-expression proteins will be found to have important roles in the brain. By increasing specificity, co-immunoprecipitation method will be a more powerful tool to analyze protein-protein interactions and their molecular mechanisms.

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