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MONOCLONAL ANTIBODIES IMMUNOPRECIPITATING ω-CONOTOXIN-SENSITIVE CALCIUM CHANNEL MOLECULES RECOGNIZE TWO NOVEL PROTEINS LOCALIZED IN THE NERVOUS SYSTEM

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Two monoclonal antibodies raised against brain synaptic membranes immunoprecipitated siginificant fractions of the brain ω-conotoxin receptor (probable ω-conotoxin-sensitive calcium channels) solubilized with digitonin. These antibodies recognized different proteins of 36 kDa and 28 kDa, respectively. Immunoblot analysis of fractions obtained by sucrose gradient centrifugation suggested that these two proteins were not subunits of the ω-conotoxin receptor but were bound to it. These proteins were found to be conserved at least from an amphibian to mammals, and to be present in the nervous system and adrenal medulla among the tissues examined.

Multiple voltage-sensitive calcium channels with different voltage dependence, gating kinetics and pharmacological properties are present in both the peripheral and central nervous system of vertebrates (1,2). The localizations of these different calcium channels in the brain are not well understood, but their limited distribution, together with their different voltage dependence and gating kinetics, should contribute to determining spatio-temporal gradients of intracellular Ca^{2+} concentration that regulate both short- and long-term local cellular responses.

Pharmacological properties are useful criteria for classifying voltage-sensitive calcium channels. ω-Conotoxin GVIA (GVIA) specifically blocks some neuronal calcium channels (3). Very recently, we showed that most dihydropyridine (DHP) receptor molecules are distinct from GVIA receptor molecules in mammalian brain (4). This finding is consistent with recent reports that GVIA does not block neuronal DHP-sensitive calcium channel currents (5-8). We attempted to generate monoclonal antibodies (MAbs) against the brain GVIA receptor using synaptic plasma membranes (SPMs) as antigen and obtained two MAbs that immunoprecipitated significant portions of the GVIA receptor solubilized from brain membranes. The main antigens of these two MAbs were two small proteins localized in the nervous system and related tissues. Probably these proteins are bound to some of the GVIA receptors.

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MATERIALS AND METHODS

Immunoprecipitations of GVIA Receptors: GVIA receptors in lysed P2 from bovine and chick brains were prelabeled with 0.3 nM [^{3}H]monopropionyl-GVIA ([^{3}H]Pr-GVIA), solubilized with digitonin, and subjected to immunoprecipitation using anti-mouse IgG-Sepharose as described previously (4).

Preparation of MAbs: SPMs were prepared from bovine cerebral cortex and rat forebrain (9) in the presence of protease inhibitors (5 mM EDTA, 1 mM iodoacetamide, 0.3 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin and 1 μM pepstatin A). BALB/c mice were immunized by intraperitoneal injection of SPMs at two-week intervals (500 μg protein in 0.1 ml of 10 mM Hepes-NaOH, pH 7.4) emulsified with an equal volume of Freund's complete adjuvant (1st injection) or incomplete adjuvant (subsequent injections). Three days after the 4th injection, spleen cells were removed and fused with myeloma cells (PAI) (4). Hybridoma culture supernatants were screened for MAbs that immunoprecipitated solubilized GVIA receptors prelabeled with ([^{3}H]Pr-GVIA) as described above. SPM-1 and SPM-2 (both IgG_1) were obtained from mice immunized with bovine and rat SPMs, respectively. MAbs were purified from ascites fluids by affinity chromatography on anti-mouse IgG-Sepharose.

Affinity Chromatography on MAb-Sepharose: Purified SPM-1 or SPM-2 was coupled to CNBr-activated Sepharose 4B (3 mg MAb/ml gel). All following procedures were carried out at 0-4°C. All the solutions used contained the mixture of protease inhibitors described above unless otherwise stated. The lysed P2 fraction (10) from bovine cerebral cortex (120 mg protein) was extracted for 30 min with 60 ml of Solution A (20 mM Tris-HCl-0.2 M NaCl, pH 7.4) containing 1% digitonin. The mixture was centrifuged at 100,000 g for 1 h, and the supernatant was diluted twice with an equal volume of Solution A, and gently rotated for 1.5 h with 3.5 ml of SPM-1- or SPM-2-Sepharose 4B. Most of the supernatant was removed by centrifugation at 1,000 g for 1 min, and the resin was packed into a plastic column and washed first with 30 ml of Solution A containing 0.1% digitonin, and then with 30 ml of distilled water (without protease inhibitors). The bound material was eluted with 1% sodium dodecyl sulfate (SDS) in 50 mM Tris-HCl, pH 6.8 (without protease inhibitors) at room temperature. SDS-PAGE (polyacrylamide gel electrophoresis) was carried out by the method of Laemmli (11) using 5-15% gradient gels.

RESULTS

Immunoprecipitation of GVIA Receptors by the MAbs

Figure 1 illustrates the immunoprecipitation of GVIA receptors solubilized from bovine and chick brain membranes by ascites fluids containing SPM-1 or SPM-2. The maximal precipitations by SPM-1 and SPM-2 were 31.2 ± 0.8% (mean ± S.D., n=3), and 23.8 ± 1.0% (n=3), respectively. Simultaneous addition of both MAbs at concentrations that gave maximal precipitation when added separately did not significantly increase the precipitation (31.6 ± 1.9%, n=3) (see Fig. 1). In some experiments, the GVIA receptor remaining in the supernatant after treatment with one MAb was further precipitated with the other MAb. In this case, total precipitation never exceeded that with SPM-1 alone (data not shown). These results indicate that all the GVIA receptors precipitated by SPM-2 were included in those precipitated by SPM-1. These two MAbs also precipitated GVIA receptors solubilized from chick brain membranes, although the maximal precipitations were somewhat lower (23.4 ± 1.0% for SPM-1, and 20.7 ± 1.2% for SPM-2, n=3) (Fig. 1). As with bovine brain membranes, the precipitation was not increased by simultaneous addition of both MAbs

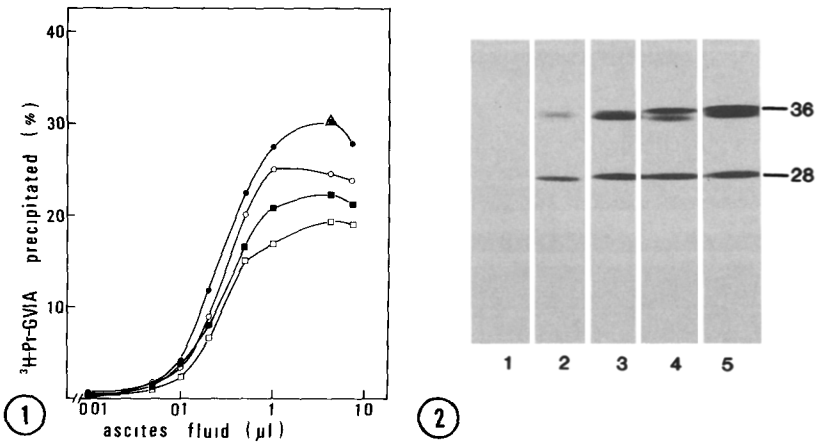

Fig. 1. Immunoprecipitation of the GVIA receptor solubilized from brain lysed P2 fractions. Immunoprecipitations by SPM-1 (filled symbols) or SPM-2 (open symbols) are expressed as percentages of the total GVIA receptor used. Circles and squares represent results for bovine and chick brain membranes, respectively. The triangle indicates the immunoprecipitation of bovine brain GVIA receptors with 4 μl of SPM-1 and 1 μl of SPM-2 added simultaneously. Typical examples are shown.

Fig. 2. Distribution of the antigens for SPM-1 and SPM-2 in various animals. Lysed P2 fractions from the brains of various animals were fractionated by SDS-PAGE (10 μg protein/sample) and then immunoblotted (4) with a mixture of SPM-1 and SPM-2. Lane 1, trout; lane 2, bullfrog; lane 3, chick; lane 4, rat; lane 5, bull. Values on the right represent the molecular masses (in kDa) of the components detected.

(data not shown). SPM-1 and SPM-2 caused negligible immunoprecipitation (3-6% of the total, with 1-4 μl of either antibody , n=3) of bovine brain DHP receptors prelabeled with [^{3}H]PN200-110 and solubilized with digitonin from the same membranes (4).

Antigens for the MAbs

In immunoblot analysis of lysed P2 fractions from rat and bovine brains, components (proteins) with molecular masses of 36 kDa and 28 kDa were detected by SPM-1 and SPM-2, respectively (Fig. 2). The same components were detected when SDS-PAGE was carried out under non-reducing conditions (in the presence of 10 mM N-ethylmaleimide) and reducing conditions (in the presence of 20 mM dithiothreitol). Sometimes the 36 kDa protein formed a closely spaced doublet (see Fig. 2), probably indicating the presence of more than one isoform. Even when large amounts (>200 μg protein) of brain membranes were blotted, no cross-reactions were observed between SPM-1 and the 28 kDa component or between SPM-2 and the 36 kDa component (cf. Fig. 4), indicating that the two MAbs recognize distinct proteins.

Purification of the Antigens for the MAbs

When a digitonin extract of bovine or rat brain lysed P2 was applied to a column of SPM-1-Sepharose, most of the 36 kDa protein was retained as shown

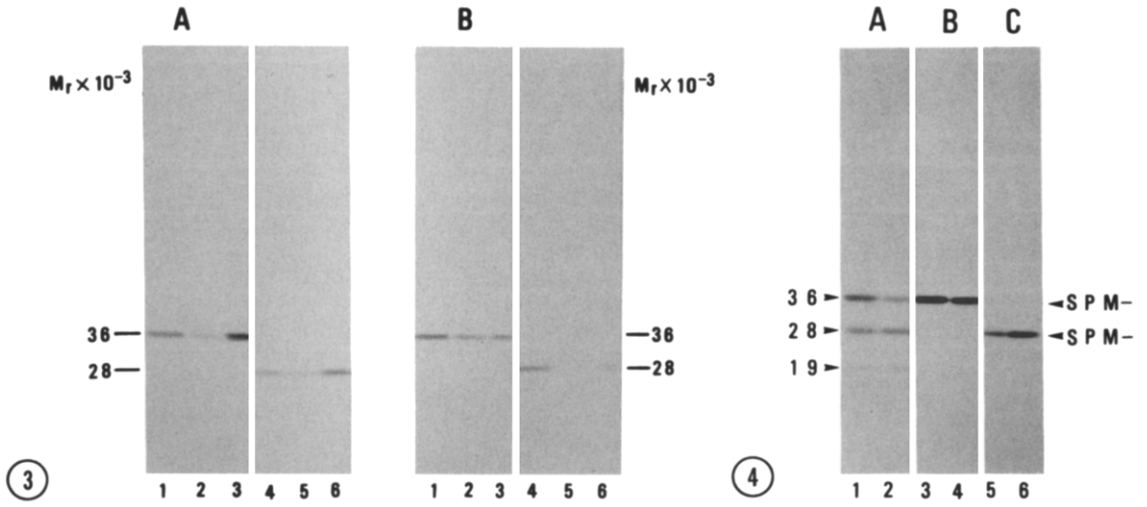

Fig. 3. Affinity chromatography of a digitonin extract of the lysed P2 fraction from bovine brain on MAb-Sepharose. After SDS-PAGE, samples were analyzed by immunoblotting. For details, see MATERIALS AND METHODS. (A) SPM-1-Sepharose. Lanes 1-3, immunoblot with SPM-1; lanes 4-6, immunoblot with SPM-2. Lanes 1 and 4, digitonin extract; lanes 2 and 5, unadsorbed fraction; lanes 3 and 6, SDS-eluate. (B) SPM-2-Sepharose. Samples were exactly as for A. Poor elution of the 28 kDa protein with SDS (lane 6) was due to its firm binding to SPM-2.

Fig. 4. SDS-eluates from SPM-1- and SPM-2-Sepharose.
(A) Amido Black 10B staining of blots. (B) Immunoblot with SPM-1. (C) Immunoblot with SPM-2. Lanes 1, 3 and 5: SDS-eluate from SPM-1-Sepharose; lanes 2, 4 and 6: SDS-eluate from SPM-2-Sepharose.

by immunoblotting of the unadsorbed fraction. A portion of the 28 kDa protein was also retained (Fig. 3A). Conversely, most of the 28 kDa protein and some of the 36 kDa protein were retained on a column of SPM-2-Sepharose (Fig. 3B). The partial retention of either the 28 kDa protein or the 36 kDa protein was not due to the use of insufficient MAb resins, as the retentions of these proteins did not increase on use of increased amounts of the resins. The bound proteins were eluted with 1% SDS at room temperature (Figs. 3A and B, lane 3). The SDS-eluate from SPM-1-Sepharose contained three major proteins of 36 kDa, 28 kDa and 19 kDa (Fig. 4A). Three components with these molecular masses were obtained by affinity chromatography of the same digitonin extract on SPM-2-Sepharose (Fig. 4B). The 36 kDa and 28 kDa components were the antigens for SPM-1 and SPM-2, respectively, as they reacted with these respective antibodies (Figs. 4A and B). However, the 19 kDa protein did not react with either SPM-1 or SPM-2 (Fig. 4). The proteins purified by immunoaffinity chromatography on SPM-1- and SPM-2-Sepharose gave similar patterns on two-dimensional electrophoresis (data not shown), so the 36 kDa, 28 kDa and 19 kDa proteins obtained from SPM-1-Sepharose were probably identical or closey resembled the 36 kDa, 28 kDa and 19 kDa proteins,

respectively, obtained from SPM-2-Sepharose. None of these three proteins was obtained by affinity chromatography of digitonin extracts of bovine lysed P2 on normal mouse IgG-Sepharose.

None of the partial amino acid sequences of several peptides derived from the 36 kDa or 28 kDa protein digested with Staphylococcus V8 protease showed significant homology to any protein so far sequenced (Y. S. et al., unpublished observations). Comparison of the peptides of these two proteins digested with lysyl endopeptidase by SDS-PAGE did not show any peptides common to both proteins (data not shown). Thus, the 36 kDa and 28 kDa proteins do not resemble each other structurally. This is consistent with the absence of cross-reactivity of SPM-1 with the 28 kDa protein or of SPM-2 with the 36 kDa protein (Fig. 4). On SDS-PAGE of large amounts (20-50 μg protein) of the SDS-eluates from SPM-1- and SPM-2-Sepharose, several faintly stained bands of high molecular masses (50-300 kDa) were detected in addition to the three components described above (data not shown). These bands should contain subunits of the GVIA receptor, but they have not yet been identified.

Relationship between the Antigens and the GVIA Receptor

GVIA receptors in lysed P2 prelabeled with [^{3}H]Pr-GVIA were solubilized with digitonin and subjected to sucrose density gradient centrifugation (Fig. 5). The GVIA receptor sedimented as a single peak, as described previously for

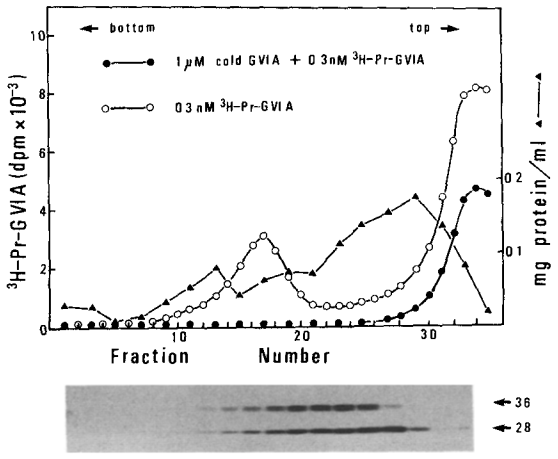

Fig. 5. Sucrose density gradient centrifugation of a digitonin extract of the rat brain lysed P2 fraction prelabeled with [^{3}H]Pr-GVIA. A digitonin extract (1 ml) of the lysed P2 fraction of rat brain prelabeled with 0.3 nM [^{3}H]Pr-GVIA in the presence (filled circles) or absence (open circles) of 1 μM unlabeled GVIA was fractionated by sucrose gradient (5-20%, w/v, in the presence of 0.1% digitonin) centrifugation as described previously (10). Fractions of 1 ml were collected, and the radioactivity of 0.1 ml aliquots of each fraction was determined. Samples of 50 μl of each odd number fraction were subjected to SDS-PAGE and immunoblotting with SPM-1 and SPM-2 (bottom).

unlabeled GVIA receptor (10). The radioactivities found at or near the top of the gradient probably represent unbound [^{3}H]Pr-GVIA, as they were not retained on a polyethyleneimine-coated glassfiber filter on filtration under reduced pressure (cf. 10). The peak of [^{3}H]Pr-GVIA disappeared when lysed P2 was incubated with 1 μM unlabeled GVIA before addition of [^{3}H]Pr-GVIA (Fig. 5), indicating that it represents [^{3}H]Pr-GVIA specifically bound to its receptors. Immunoblot analysis of gradient fractions showed that little of the 36 kDa or 28 kDa protein was associated with GVIA receptors (Fig. 5). Figure 5 also indicates that the 36 kDa and 28 kDa proteins did not move together. For instance, fraction 29 contained little of the 36 kDa protein, but an appreciable amount of the 28 kDa protein. Moreover, the 36 kDa protein was most abundant in fraction 23, while the 28 kDa protein was most abundant in fraction 27.

Distribution of the Two Proteins

We examined the distribution of these antigens in the brains of various animals by immunoblotting of brain membranes. The results (Fig. 2) showed that the 36 kDa and 28 kDa proteins are present in the brains of at least the frog, chick and mammals, but not in trout brain. The molecular masses of the reacting components in these animal brains were the same, suggesting that their antigens were similar. Neither the 36 kDa protein nor the 28 kDa protein was detected in the soluble fraction prepared in the presence or absence of 10 mM EDTA (data not shown), excluding the possibility that these two proteins are annexins, a group of proteins that bind to membranes in a Ca^{2+}-dependent manner (12). Immunoblots of various tissues indicated that the two proteins are localized in the nervous system and probably also in paraneurons such as adrenal medulla (Fig. 6). The relationship between the 36 kDa protein and a 230 kDa component detected in rat kidney is unknown.

DISCUSSION

In this paper, we report two novel proteins associated with some GVIA receptors in the brain. Several possibilities can be considered about the relatioship of these proteins with the GVIA receptor.

First, these proteins may be subunits of the GVIA receptor (some calcium channels). However, these proteins were mainly separated from the GVIA receptor by sucrose density gradient centrifugation (Fig. 5). There is a remote possibility that these two proteins were originally entirely associated with the GVIA receptor, but were dissociated during the experimental procedures used. However, the amounts of the 36 kDa and 28 kDa proteins estimated from their yields on affinity chromatography on SPM-1- and SPM-2-Sepharose indicate that they both constituted about 1/1,500-1/2,000 of the total protein in the lysed P2, while the amount of the GVIA receptor calculated from the number of high-affinity binding sites for [^{3}H]Pr-GVIA in

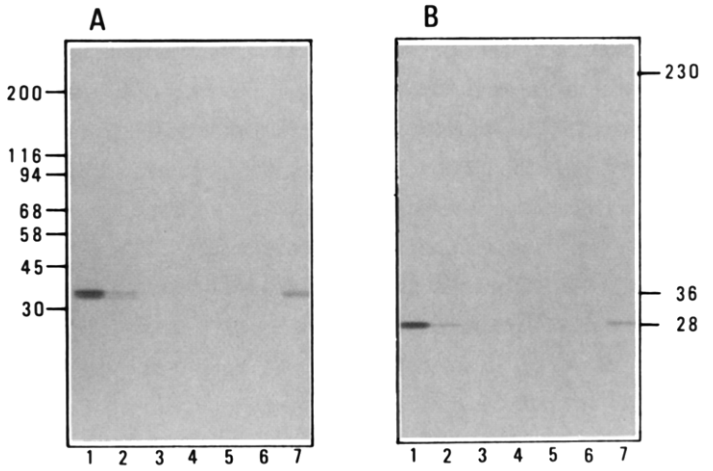

Fig. 6. Tissue distribution of the antigens for SPM-1 and SPM-2 analyzed by immunoblotting. Lysed P2 fractions (lanes 1-4 and 7) and microsomes (lanes 5 and 6) were subjected to SDS-PAGE (10 μg protein for brain, 100 μg protein for other tissues) and then immunoblotted. Values on the left and right are the molecular masses (in kDa) of marker proteins and the components detected, respectively. (A) Immunoblot with SPM-1. Lanes 1-6, rat brain, superior cervical ganglion, liver, kidney, skeletal muscle and cardiac muscle, respectively; lane 7, bovine adrenal medulla. (B) Immunoblot with SPM-2. Samples were the same as for A.

the lysed P2 (10), assuming that the molecular mass of the GVIA receptor is 500 kDa, would be about 1/6,000 of the total. Thus, the 36 kDa and 28 kDa proteins are probably more abundant than the GVIA receptor, so it is very unlikely that these two proteins are unique subunits of the GVIA receptor. Moreover, the 36 kDa protein is distinct from the components of 34 kDa and 37 kDa identified by photoaffinity labeling of the GVIA receptor in rat and bovine brain membranes, respectively (10,13), as its molecular mass was the same in rat and bovine brains (see Fig. 2).

Second, the two proteins would contain similar epitopes to those present on a population of GVIA receptor molecules. These epitopes could be recognized by SPM-1 and SPM-2, resulting in precipitation of solubilized GVIA receptor molecules as observed in the present experiments. The fact that components of high molecular mass were not observed in immunoblots of brain membranes might simply be due to irreversible loss of reactivity with SPM-1 and SPM-2 of the epitopes on the GVIA receptor in SDS. However, the finding that some of the 28 kDa and 36 kDa proteins bind to SPM-1- and SPM-2-Sepharose, respectively, cannot be explained by the above assumption, as there was no indication of cross-reactivity between these combinations. Thus, some of the 36 kDa and 28 kDa proteins are probably bound to a population of GVIA receptors directly or indirectly. Although the functions of these two proteins are unknown, they may regulate GVIA-sensitive calcium channels. The

fact that only about 1/4-1/3 of the solubilized GVIA receptor was immunoprecipitated by SPM-1 or SPM-2 may indicate heterogeneity of the regulatory mechanisms for GVIA-sensitive calcium channels in the brain. Recently, a MAb that recognizes a brain protein was shown to immunoprecipitate about 1/4 of the solubilized GVIA receptor (14). Although this protein also appears to be bound to the GVIA receptor, its size (58 kDa) indicates that it probably differs from the 36 kDa and 28 kDa proteins described here.

On affinity chromatography of a digitonin extract on SPM-1- and SPM-2-Sepharose, a component of 19 kDa was reproducibly obtained besides the 36 kDa and 28 kDa proteins. The relatioship of this component with the 36 kDa and 28 kDa proteins is not known, but it may also be bound to GVIA receptors or to the 36 kDa and/or 28 kDa protein.

The conservation of the 36 kDa and 28 kDa proteins during evolution, at least from an amphibian to mammals, and their localization in the nervous system and paraneurons strongly suggest these proteins play fundamental roles in the nervous system. Probably only very small portions of the 36 kDa and 28 kDa proteins are associated with GVIA receptors, so these proteins may have functions other than in relation to GVIA-sensitive calcium channels.

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REFERENCES

1. Hess, P. (1990) Annu. Rev. Neurosci. 13, 337-356.
2. Tsien, R.W. and Tsien, R.Y. (1990) Annu. Rev. Cell Biol. 6, 715-760.
3. Gray, W.R. and Olivera, B.M. (1988) Annu. Rev. Biochem. 57, 665-700.
4. Hayakawa, N., Morita, T., Yamaguchi, T., Mitsui, H., Mori, K.J., Saisu, H. and Abe, T. (1990) Biochem. Biophys. Res. Commun. 173, 483-490.
5. Kasai, H., Aosaki, T. and Fukuda, J. (1987) Neurosci. Res. 4, 228-235.
6. Aosaki, T. and Kasai, H. (1989) Pflug. Arch. 414, 150-156.
7. Plummer, M.R., Logothetis, D.E. and Hess, P. (1989) Neuron 2, 1453-1463.
8. Regan, L.J., Sah, D.W. and Bean, B.P. (1991) Neuron 6, 269-280.
9. Jones, D.H. and Matus, A.I. (1974) Biochim. Biophys. Acta 356, 276-287.
10. Yamaguchi, T., Saisu, H., Mitsui, H. and Abe, T. (1988) J. Biol. Chem. 263, 9491-9498.
11. Laemmli, U.K. (1970) Nature 227, 680-685.
12. Geisow, M.J. and Walker, J. H. (1986) Trends Biochem. Sci. 11, 420-423.
13. Abe, T. and Saisu, H. (1987) J. Biol. Chem. 262, 9877-9882.
14. Takahashi, M., Arimatsu, Y., Fujita, S., Fujimoto, Y., Kondo, S., Hama, T. and Miyamoto, E. (1991) Brain Res. 551, 279-292.