

Validation of Anti-CSP α , SNAP25, Tyrosine Hydroxylase, Ubiquitin, Cleaved Caspase 3, and pSer PKC Motif Antibodies for Utilization in Western Blotting

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There are many commercial antibodies with little information provided by their suppliers as to their reliability. Accordingly, commercial antibodies require proper validation before being used in scientific research. In this study, we validated several commercial antibodies, including anti-CSP α , SNAP25, tyrosine hydroxylase, ubiquitin, cleaved caspase 3, and pSer PKC motif. Anti-CSP α , SNAP25, and tyrosine hydroxylase antibodies could detect their endogenous target proteins with some degree of cross-reactivity. Furthermore, clear SNAP25 staining was observed with SNAP25 antibody. Antibodies directed against ubiquitin, cleaved caspase 3, and pSer PKC motif could detect poly-ubiquitination, apoptosis, and phosphorylation, respectively.

Key words: validation, antibody, Western blotting

I. Introduction

Recently, there has been growing attention regarding the reliability of commercial antibodies, given that certain recent publications have faced the issue of reproducibility [1, 3, 4]. According to Hewitt *et al.*, the Histochemical Society advocated standards of practice for validation of immunohistochemical assays by using appropriate control [2]. Accordingly, antibodies require proper validation before being used in scientific research. As our group studies the protection system of dopaminergic synaptic terminals through signal transduction, the aim of the present study was to evaluate the reliability of the antibodies we use, including cysteine string protein alpha (CSP α), synaptosomal-associated protein 25 (SNAP25), tyrosine hydroxylase (TH), ubiquitin (Ub), cleaved caspase 3, and phospho-Serine protein kinase C motif (pSer PKC motif).

II. Materials and Methods

Reagents

MG132 was obtained from WAKO (Wako Pure Chemical Industries, Osaka, Japan) and 12-*O*-Tetradecanoylphorbol 13-acetate (TPA) was obtained from Sigma-Aldrich (St. Louis, MO).

Antibodies

Anti-TH (T-1299) and anti- β -tubulin from Sigma-Aldrich. Anti-Ub (sc-8017), rabbit IgG (sc-2027), and mouse IgG (sc-2025) from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-pSer PKC motif (#2261) and anti-cleaved caspase3 (#9661) from Cell Signaling (Danvers, MA); anti-CSP α (ab90499) and anti-SNAP25 (ab41455) from Abcam (Cambridge, UK); anti-SNAP25 (MAB331) from Millipore (Billerica, MA); Horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse secondary antibodies from Jackson ImmunoResearch Inc. (West Grove, PA). It is noted that anti-CSP α (ab90499) and anti-SNAP25 (ab41455) antibodies were raised in rabbit and anti-TH (T-1299) and anti-SNAP25 (MAB331) antibodies were raised in mouse.

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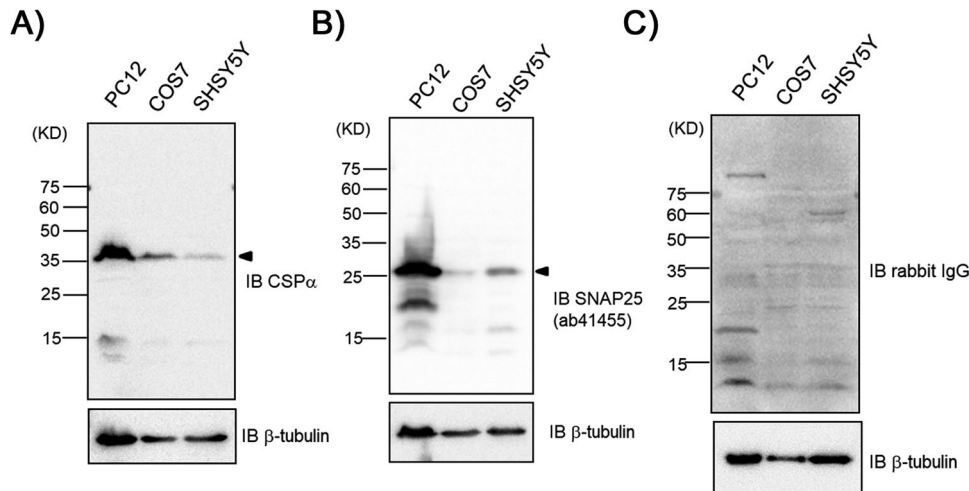


Fig. 1. Immunoblot with antibodies raised in rabbits against CSP α and SNAP25. **A)** Immunoblot for CSP α in PC12, COS7, and SHSY5Y cells. A band at 35 kDa was detected in all cells, and lower molecular weight, non-specific bands were also found in PC12 cells. Arrowhead indicates the bands corresponding to CSP α . **B)** Immunoblot for SNAP25 (ab41455) in PC12, COS7, and SHSY5Y cells. A band at 25 kDa was detected in all cells, and lower molecular weight, non-specific bands were also identified for all cells. Arrowhead indicates the bands of SNAP25. **C)** Immunoblot using normal rabbit IgG, which is a negative control, in PC12, COS7, and SHSY5Y cells. Several bands were detected in all cells.

Sample preparation

Homogenization buffer containing 150 mM NaCl, 10 mM ethylene glycol tetracetic acid, 2 mM ethylenediamine tetracetic acid, 10 mM HEPES, pH 7.4, protease-inhibitor cocktail (Nacalai Tesque, Kyoto, Japan), and a phosphatase-inhibitor cocktail (Nacalai Tesque).

PC12, COS7, and SHSY5Y cells were homogenized and sonicated in homogenization buffer. Samples were centrifuged ($200,000 \times g$ for 15 min at 4°C) and the supernatant was then combined with 3X loading buffer and incubated at 95°C for 5 min.

Immunoblotting

Samples derived from cells were electrophoresed on a 12.5% Tris-Glycine gel, and transferred to a polyvinylidene fluoride membrane, which was blocked for 1 hr with 5% skim milk powder in a solution of 0.1% Triton X-100 in TBS (TBST). Membranes were then probed with antibody at a concentration of 1:1000 in antibody diluent in TBST or normal mouse IgG and rabbit IgG at a concentration of 1:1000. Membranes were incubated overnight at 4°C. Following washing in TBST, membranes were incubated with horse radish peroxidase-conjugated anti-rabbit IgG at a concentration of 1:10000 or anti-mouse IgG at a concentration of 1:10000 in TBST for 1 hr at room temperature.

Immunocytochemistry

After fixing with 4% paraformaldehyde for 30 min at room temperature, permeabilizing with TBST for 1 hr at room temperature, and blocking with 5% normal goat serum, the PC12 and COS7 cells were incubated with anti-SNAP25 (MAB331) antibody (1:500) overnight at 4°C and Alexa Fluor 488-conjugated anti-mouse IgG antibody (1:500) for 45 min at room temperature. The immuno-

reactivity was visualized using a fluorescence microscope (BZ-9000, Keyence, Osaka, Japan).

III. Results and Discussion

To investigate the reliability of the antibodies, we performed immunoblot analyses using homogenates from PC12, COS7, and SHSY5Y cells. First, rabbit antibodies, including anti-CSP α and SNAP25, were evaluated (Fig. 1). Anti-CSP α antibody generated a band at approximately 35 kDa in PC12, COS7, and SHSY5Y cells (Fig. 1A). Anti-SNAP25 (ab41455) antibody detected several bands in PC12 and SHSY5Y cells (Fig. 1B). As rabbit IgG yielded several bands in PC12, COS7, and SHSY5Y cells (Fig. 1C), we concluded that the band at 35 kDa generated by anti-CSP α antibody reflected endogenous CSP α , and the band near 25 kDa generated by anti-SNAP25 (ab41455) antibody revealed endogenous SNAP25. As these antibodies generated non-specific bands typically originating from rabbit IgG, they may be suitable for immunoblot analysis rather than immunohistochemistry.

Second, mouse antibodies, including those against TH and SNAP25 were evaluated (Fig. 2). Anti-SNAP25 antibody (MAB331) detected a band near 25 kDa only in PC12 cells and several lower bands in all cell types (Fig. 2A). Anti-TH antibody generated many bands, including a clear band at 60 kDa in PC12 cells, a band at 20 kDa in COS7 cells, and several bands in SHSY5Y cells (Fig. 2B). We concluded that the band at 25 kDa generated by the anti-SNAP25 antibody (MAB331) reflected endogenous SNAP25 protein, and the band at 60 kDa yielded by the anti-TH antibody showed endogenous TH. As mouse IgG detected only one band in PC12 cells (Fig. 2C), anti-SNAP25 and anti-TH antibodies detected non-specific

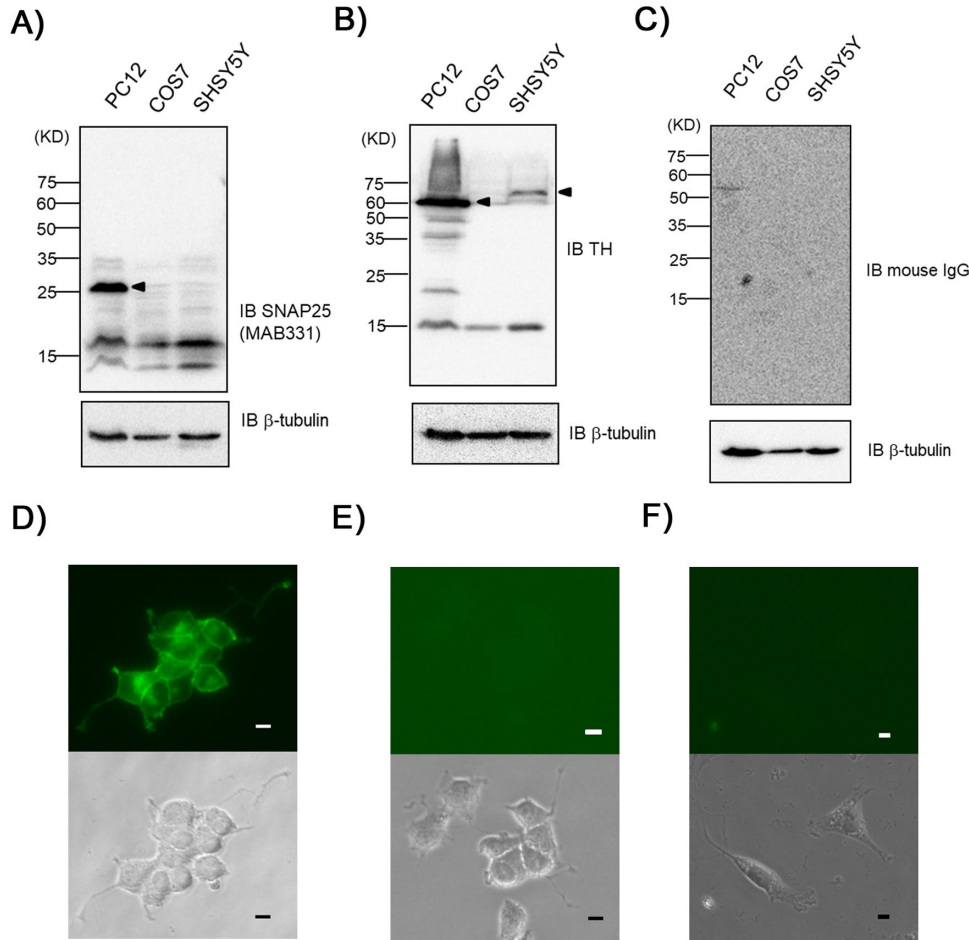


Fig. 2. Immunoblot using antibodies raised in rabbits against SNAP25 and TH and immunocytochemistry using SNAP25 antibody. **A)** Immunoblot for SNAP25 (MAB331) in PC12, COS7, and SHSY5Y cells. A band at 25 kDa was detected only in PC12 cells, and lower molecular weight, non-specific bands were identified in all cells. Arrowhead indicates the bands of SNAP25. **B)** Immunoblot for TH in PC12, COS7, and SHSY5Y cells. Many bands, including a distinct band at 60 kDa, were detected in PC12 cells. Two bands at 60 kDa and 15 kDa were detected in SHSY5Y cells. A band at 15 kDa was detected in COS7 cells. Arrowheads indicate the bands corresponding to TH. **C)** Immunoblot using normal mouse IgG, which is a negative control, in PC12, COS7, and SHSY5Y cells. One band was detected only in PC12 cells. **D)** Immunocytochemistry for anti-SNAP25 (MAB331) antibody in PC12 cells. Clear SNAP25 staining was observed in the cytosol of PC12 cells. Bar = 10 μm. **E)** Immunocytochemistry for Alexa Fluor 488-conjugated anti-mouse IgG antibody in PC12 cells as a negative control. No staining was observed. Bar = 10 μm. **F)** Immunocytochemistry for anti-SNAP25 (MAB331) antibody in COS7 cells. No staining was observed. Bar = 10 μm.

bands generated by these antibodies. Next, we performed the immunocytochemistry as to anti-SNAP25 (MAB331) antibody in PC12 cells and COS7 cells. Clear SNAP25 staining was observed in the cytosol of PC12 cells (Fig. 2D). However, there was no staining in COS7 cells (Fig. 2F). These findings suggested that anti-SNAP25 (MAB331) antibody could be used for the immunoblot and immunocytochemistry, even though there was some non-specific reaction in the immunoblot in COS7 cells. While antibody without any non-specific reactions is ideal, specific antibody is often unavailable from commercial sources. Therefore, considering various data, such as immunoblot, immunocytochemistry, immunohistochemistry with positive and/or negative control study, and *in situ* hybridization study, the immunoreaction could be understood as real positive reaction synthetically.

Finally, antibodies recognizing functions such as apoptosis, phosphorylation, and ubiquitination were evaluated (Fig. 3). Anti-cleaved caspase 3 antibody detected three bands, and serum-starvation, which induces apoptotic cell death, increased the intensity of bands in PC12 cells, suggesting that these bands reflect appropriately cleaved caspase 3 (Fig. 3A). As the manufacturer's datasheet indicates that anti-cleaved caspase 3 detects 17 and 19 kDa bands, the lowest band may be non-specific. Anti-Ub antibody yielded two lower bands and higher molecular weight smear bands in PC12 cells (Fig. 3B). The band near 10 kDa is monomeric Ub and the upper smear bands may reflect various poly-ubiquitinated proteins. Moreover, MG132, which is a proteasome inhibitor, increased the intensity of the upper bands, suggesting that this anti-Ub antibody could detect both poly-ubiquitin and mono-ubiquitin. The

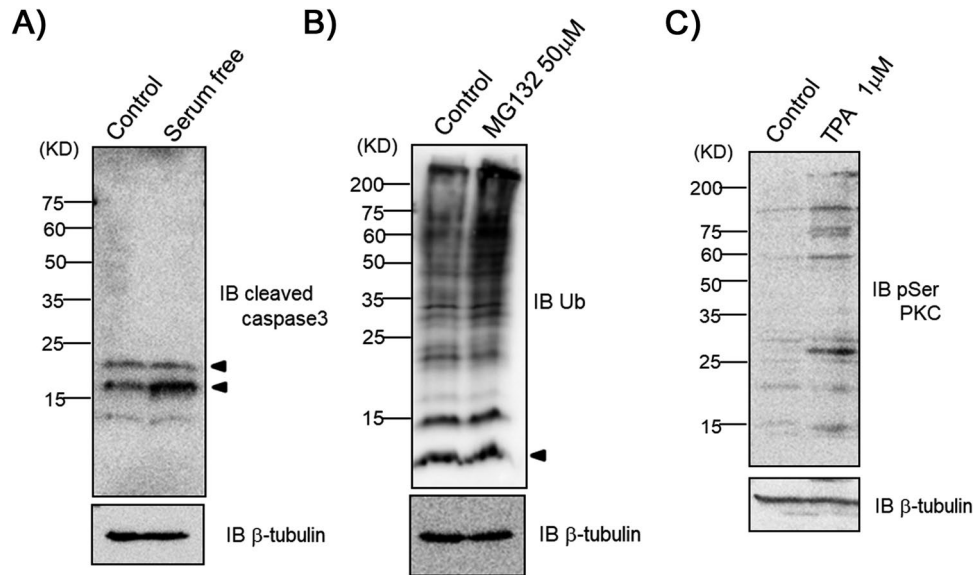


Fig. 3. Validation of antibodies recognizing functions including apoptosis, ubiquitination, and phosphorylation. **A)** Immunoblot for cleaved caspase 3 in PC12 cells. PC12 cells incubated with and without serum overnight were immunoblotted using anti-cleaved caspase 3 antibody. Arrowheads for both 17 and 19 kDa bands indicate cleaved caspase 3. **B)** Immunoblot for ubiquitin in PC12 cells. PC12 cells incubated with or without MG132, 50 μ M for 2 hr, were immunoblotted using anti-ubiquitin antibody. Arrowhead at 8 kDa indicates monomeric Ub. **C)** Immunoblot for pSer PKC motif in COS7 cells. COS7 cells incubated with or without TPA, 1 μ M for 1 hr, were immunoblotted using anti-pSer PKC motif antibody.

anti-pSer PKC motif antibody generated many bands in COS7 cells. Furthermore, TPA, which is a PKC stimulator, increased the intensity of some of the bands (Fig. 3C), suggesting that the anti-pSer PKC motif antibody could adequately detect phosphorylation. In conclusion, anti-CSP α , SNAP25, and TH antibodies could detect their endogenous target proteins with some cross-reactive proteins. As these antibodies generated non-specific bands, they are suitable for immunoblot analysis. Anti-Ub, cleaved caspase 3, and pSer PKC motif antibodies could detect poly-ubiquitination, apoptosis, and phosphorylation, respectively.

IV. Conflict of Interest

The authors declare no conflicts of interest associated with this manuscript.

V. References

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