Monoclonal Antibody Register

Monoclonal Antibody BF-06 against the Heavy Chain of Clathrin

L. MACŮREK, M. ZÍKOVÁ, E. DRÁBEROVÁ, P. DRÁBER

Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Background

Clathrin is a ubiquitously expressed protein which polymerizes into a coat-like lattice on the cytoplasmic surface of cellular membranes (Brodsky et al., 1988). Formation of a polyhedral lattice is enabled by self-assembly of clathrin triskelions, which are composed of three copies of clathrin heavy chain (~180kDa) and three light chain (~30kDa) subunits. Clathrin is a major component of coated vesicles (Pearse et al., 1975), from which it dissociates at higher ionic strength or at basic pH (Liu et al., 1995). Reassociation of purified clathrin *in vitro* occurs after lowering the pH. Clathrin polymerization results in membrane invagination and is dynamically regulated by light chains *in vivo*. Clathrin-coated vesicles are responsible for both the receptor-mediated endocytosis and the secretory vesicle budding from the *trans*-Golgi network. Interaction of clathrin with the membrane is indirect and is mediated by adaptor proteins (AP 1–4) specific for different cellular compartments (Kirchhausen, 1999). Immunofluorescence with anti-clathrin antibodies results in a punctuate staining of the vesicles in the perinuclear region of interphase cells. In mammalian cells, localization of clathrin on microtubular arrays of mitotic spindles was also reported (Okamoto et al., 2000).

Description of the antibody BF-06

Production

A hybridoma cell line producing the BF-06 antibody was selected by immunoblotting screening of supernatants in the course of preparation of hybridomas producing monoclonal antibodies against the structure-specific recognition protein SSRP1. BALB/c mice were immunized with a recombinant fragment (a.a. 150–413) of mouse SSRP1, and spleen cells were fused with Sp2/0 myeloma cells. Details of the fusion, screening, cloning and production of ascitic fluid have been described previously (Dráber et al., 1988; Viklický et al., 1982). In immunoblotting, the antibody BF-06 reacted weakly with the recombinat fragment, but unexpectedly strong reaction was detected with preparations from brain tissues. The specificity of the antibody was therefore studied in more detail.

Specificity

Immunoblotting analysis performed using the BF-06 antibody with low-speed supernatant (40,000 *g*, 20 min, 4°C) of porcine brain tissue extract showed a specific reaction with a protein of relative electrophoretic mobility around 180 kDa (Fig. 1A, lane 1). After high-speed centrifugation of the low-speed supernatant (100,000 *g*, 60 min, 4°C), the antibody gave a faint staining of the 180 kDa protein in the supernatant (Fig. 1A, lane 2), but a strong reaction was detected in the membrane-enriched pellet (Fig. 1A, lane 3). No cross-reaction was observed with other abundant brain proteins such as tubulin, proteins of neurofilament triplet or high-molecular weight microtubule-associated proteins.

After binding to anti-mouse antibody immobilized on CNBr-activated Sepharose 4B, the BF-06 antibody effectively immunoprecipitated the 180 kDa protein from porcine brain extract as verified by SDS-PAGE. The immunoprecipitated protein band was cut out from the gel that was shortly stained by Coomassie Brilliant Blue R-250, digested by trypsin and subjected to the mass spectrometric analysis (Dr. P. Novák, Laboratory of Molecular Structure Characterization, Institute of Microbiology, Prague). Mass spectrum was measured using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) in a mass spectrometer BIFLEX II (Bruker-Franzen Analytik, Bremen, Germany) equipped with a nitrogen laser (337 nm). Twenty-two different peptide fragments exactly matched the sequence of the bovine clathrin heavy chain (Accesion number P49951 in the Swiss-Prot Sequence Database). The analysed peptides covered 20.1% of the total protein sequence of the clathrin heavy chain, and were located in different regions of the molecule (Table 1).

Received November 20, 2003. Accepted December 2, 2003.

This work was supported in parts by grant from the Grant Agency of the Academy of Sciences of the Czech Republic (IBS 5052301) and from the Ministry of Education of the Czech Republic (LN00A026).

Corresponding author: Pavel Dráber, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Tel.: (+420) 241 062 632; Fax: (+420) 241 062 758; e-mail: paveldra@biomed.cas.cz.

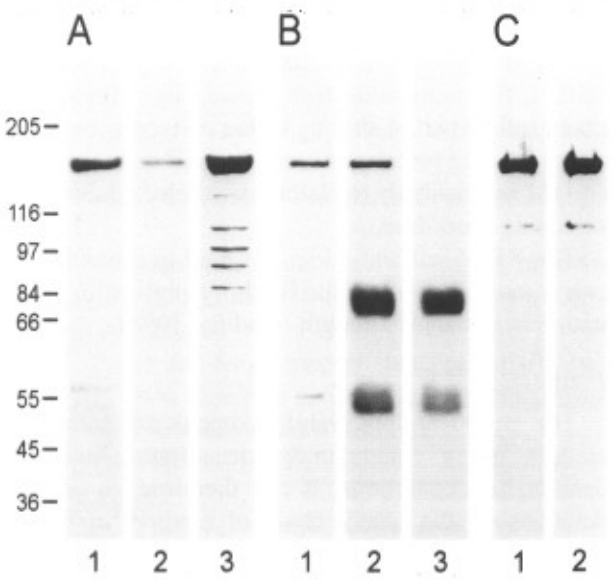

Fig. 1. The specificity of antibody BF-06 to the heavy chain of clathrin as determined by immunoblotting. **A**, porcine brain tissue extracts. Low-speed supernatant (lane 1), high-speed supernatant (lane 2) and high-speed membrane pellet (lane 3) immunostained with the BF-06 antibody. The same amount of proteins (7 μg) was loaded into each lane. **B**, immunoprecipitation of porcine brain low-speed supernatant with the BF-06 antibody followed by immunostaining with commercial X22 antibody against the heavy chain of clathrin. Proteins remaining after precipitation (lane 1), immuno-precipitated proteins (lane 2) and proteins bound to immobilized anti-mouse antibody (lane 3). **C**, purified bovine clathrin. Immunostaining with the BF-06 antibody (lane 1) and commercial antibody against the heavy chain of clathrin (lane 2). The same amount of proteins (1 μg) was loaded into each lane. Separation on 7.5% SDS-PAGE. Numbers on the left margin indicate the position of molecular weight markers in kDa.

Table 1. MALDI-TOF analysis of the 180 kDa protein immunoprecipitated by the BF-06 antibody from porcine brain.

Peptide fragment	Corresponding clathrin heavy chain sequence* (amino acid position)
VGEQAQVVIIDMNDPSNPIR	44-63
SKMKAHTMTDDVTFWK	97-112
WLLLTGISAQQNR	164-176
VSQPIEGHAASFAQFK	190-205
ISGETIFVTAPHEATAGIIGVNR	298-320
NNLAGAEELFAR	355-366
KFNALFAQGNYSEAAK	367-382
SVDPTLALSVYLR	469-481
KVGYTPDWIFLLR	507-519
ALEHFTDLYDIKR	626-638
LLLPWLEAR	857-865
FLRENPYYDSR	893-903
RPLIDQVVQTALSETQDPEEVSVTVK	968-993
IVLDNSVFSEHR	1011-1022
KFDVNTSAVQVLIEHIGNLDR	1074-1094
ESYVETELIFALAK	1166-1179
LAELEEFINGPNNAHIQQVGDR	1183-1204
LLYNNVSNFGR	1216-1226
LASTLVHLGEYQAAVDGAR	1227-1245
VANVELYYR	1398-1406
QLPLVKPYLR	1444-1453
TSIDAYDNFDNISLAQR	1482-1498

*All peptides reached 100% homology with corresponding peptides from the bovine clathrin heavy chain (Accesion number P49951 in the Swiss-Prot Sequence Database).

To verify the specifity of the BF-06 antibody, material immunoprecipitated from porcine brain extract was separated by SDS-PAGE and after blotting probed with the BF-06 antibody and with commercial antibody directed against the heavy chain of porcine clathrin (Affinity Bioreagents, Golden, CO; Cat. No. MA1-065; clone X22). Both antibodies reacted with the precipitated 180 kDa protein. The reactivity of the X22 antibody with precipitated proteins is shown in Fig. 1B (lane 2). Carrier with anti-mouse antibody alone did not bind the 180 kDa protein (Fig. 1B, lane 3).

The BF-06 antibody reacted with purified bovine brain clathrin (Sigma-Aldrich, Prague, Czech Republic, Cat. No. C5823) in ELISA and immunoblotting (Fig. 1C). Epitope mapping after limited proteolysis of purified clathrin with α-chymotrypsin (Macůrek et al., 2002) revealed that the BF-06 antibody recognized a different set of polypeptides than did the commercial antibody (not shown).

Further confirmation of BF-06 specificity was obtained by immunoblotting analysis of fractions separated after sucrose density gradient centrifugation of isolated porcine brain membranes (Steer et al., 1982). Centrifugation was performed either in 0.1 M MES buffer, pH 6.5, supplemented by 1 mM EGTA and 0.5 mM $MgCl_2$, or in 0.1 M Tris-Cl buffer, pH 9.0, where clathrin dissociates from coated vesicles. SDS-PAGE revealed an enrichment of the 180 kDa protein in fraction No. 10 containing 48% sucrose (Fig. 2A), which corresponds to the density of coated vesicles (Pearse et al., 1975). This fraction gave the strongest reaction with the BF-06 antibody (Fig. 2B, panel 6.5). At higher pH the protein recognized by the BF-06 antibody dissociated from vesicles, and the immunoreactivity shifted to lower-molecular-weight fractions (Fig. 2B, panel 9.0). Commercial anti-clathrin antibody gave the same staining patterns in immunoblotting as the BF-06 antibody (Fig. 2C, panels 6.5 and 9.0). A similar shift in immunoreactivity was also observed with both antibodies when centrifugation was performed in the presence of 0.5 M NaCl.

In immunoblotting, the BF-06 antibody reacted predominantly with the 180 kDa protein in mouse brain lysate and in lysates prepared from cell lines of various

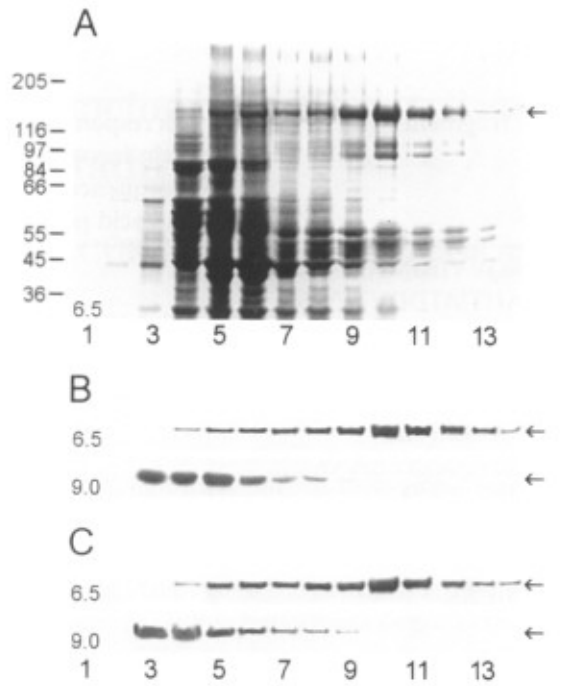

Fig. 2. The specificity of antibody BF-06 to the heavy chain of clathrin as determined by immunoblotting after sucrose-density gradient centrifugation of porcine brain membranes. Centrifugation (Beckman rotor SW-60, 250,000 *g*, 4 h, 4°C) in discontinuous gradient of 32, 40, 48, 53 and 60% (w/v) sucrose was used to enrich fractions containing coated vesicles. **A**, Coomassie blue staining of individual fractions after centrifugation in 0.1 M MES, pH 6.5. **B**, immunostaining with antibody BF-06 after centrifugation in 0.1 M MES, pH 6.5 (panel 6.5), or in 0.1 M Tris-Cl, pH 9.0 (panel 9.0). **C**, immunostaining with commercial antibody against the heavy chain of clathrin after centrifugation in 0.1 M MES, pH 6.5 (panel 6.5), or in 0.1 M Tris-Cl, pH 9.0 (panel 9.0). Separation on 7.5% SDS-PAGE. Numbers on the left margin indicate the position of molecular weight markers in kDa. Arrows on the right margin indicate the position of the 180 kDa protein. Numbers from 1 (top fraction) to 14 denote individual fractions.

species and different tissue origin: mouse neuroblastoma (Neuro2a), mouse embryonal carcinoma (P19), rat basophilic leukaemia (RBL) and human cervical adenocarcinoma (HeLa). In immunofluorescence experiments performed with P19 cells extracted with Triton X-100 and fixed with formaldehyde (Dráberová et al., 1993), the antibody gave punctuate staining in the perinuclear region and dot-like fibrillar staining (not shown). Amino acid sequence comparison did not reveal any significant similarity between mouse SSRP1 and bovine clathrin heavy chain. The weak reactivity of the BF-06 antibody with the immunizing recombinant SSRP1 fragment and strong reactivity with clathrin could reflect partial sharing of the epitope between the corresponding proteins. Alternatively, B cells producing an IgM autoantibody to clathrin was selected during the screening procedure.

The BF-06 antibody does not bind to protein L, but can be isolated from ascitic fluids by euglobulin precipitation at low ionic strength (Goding, 1996).

Properties

The BF-06 antibody (IgM, kappa) can bind brain clathrin heavy chains under denaturating and non-denaturating conditions; it can therefore be used for detection of the heavy chain of clathrin in ELISA, immunoblotting and immunoprecipitation.

Acknowledgement

We thank I. Macháčková for skillful technical assistance.

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