Monoclonal Antibodies to Discriminate the EF Hand Containing Calcium Binding Adaptor Proteins EFhd1 and EFhd2

Sebastian Brachs,¹ Christiane Lang,¹ Rolf Buslei,² Pavitra Purohit,¹ Barbara Fürnrohr,¹ Hubert Kalbacher,³ Hans-Martin Jäck,¹ and Dirk Mielenz¹

Small Ca^{2+} binding adaptor proteins of the EF hand family play important roles in neuronal and immune cell Ca^{2+} signaling. Swiprosin-1/EFhd2 (EFhd2) and Swiprosin-2/EFhd1 (EFhd1) are conserved and very homologous Ca^{2+} binding adaptor proteins of the EF hand family, with possibly redundant functions. In particular, EFhd2 has been proposed to be involved in B cell signaling and neuropathological disorders. Little is known thus far about the expression of EFhd2 on the single cell level in tissue sections or blood cells. Here we describe the generation of four specific anti-EFhd2 monoclonal antibodies. These recognize murine and human EFhd2, but not murine EFhd1, and their binding site maps to a region in the N-terminal part of EFhd2, where EFhd2 and EFhd1 differ most. Moreover, to detect EFhd1 specifically, we also generated anti-EFhd1 polyclonal antibodies, making use of a singular peptide of the N-terminal part of the protein. Using anti-EFhd2 MAb, we reveal two EFhd2 pools in B cells, one at the membrane and one cytoplasmic pool. Staining of human peripheral blood mononuclear cells shows EFhd2 expression in B cells but a ~5 fold higher expression in monocytes. Taken together, EFhd2 monoclonal antibodies will be valuable to assess the real subcellular localization and expression level of EFhd2 in healthy and diseased primary cells and tissues.

Introduction

SWIPROSIN-1/EFhd2 (EFhd2) AND SWIPROSIN-2/EFhd1 (EFhd1) are EF hand containing Ca^{2+} binding adaptor proteins, with disordered regions of low complexity, proline-rich stretches, two EF hands, and a coiled-coil domain, with an apparent molecular mass of around 35 kDa.^(1,2) EFhd1 and EFhd2 share a high degree of sequence identity and homology, suggesting at least partially redundant functions.⁽¹⁾ Both proteins bind Ca^{2+} .⁽²⁻⁴⁾ EFhd2 is highly expressed in the brain,⁽⁵⁾ has been proposed to be a calcium sensor protein,⁽³⁾ and is putatively linked to neurodegenerative diseases (see review⁽¹⁾). EFhd1 has been shown to be involved in neuronal differentiation in a cell line model.⁽²⁾ Both proteins have been linked to human schizophrenia.^(6,7) We identified EFhd2 in membrane microdomains of B cells.⁽⁸⁾ EFhd2 is also present in membrane microdomains of mouse spinal cord, but only when mice over-express a mutant, toxic gain of function form of superoxide dismutase 1, namely the G93A mutant.⁽⁹⁾ This mutant is responsible for familial amyotrophic lateral sclerosis, a chronic, progressive neuromuscular disorder.⁽¹⁰⁾ Taken

together, both EFhd1 and EFhd2 may play a role in normal and pathological brain function.

Recently human peripheral blood mononuclear cells (PBMC) have been used to assess transcriptional differences as well as alterations in proteolytic pathways between healthy donors and patients with Alzheimer's and Parkinson's disease.^(11,12) PBMC contain B cells, T cells, and innate immune cells, such as monocytes. EFhd2 is also expressed in innate immune cells, such as macrophages and NK cells, from *Drosophila* to man.^(1,13,14) Interestingly, EFhd2 has been shown to be down-regulated in PBMC of rheumatoid arthritis (RA) patients.⁽¹⁵⁾ A further study suggested that this is a proteolytic process.⁽¹⁶⁾ Thus, it will be interesting in the future to analyze EFhd2 protein expression and degradation in normal and pathological tissue and in PBMC. These analyses will provide information about mechanisms of ongoing inflammatory processes, behavioral brain disorders such as schizophrenia, and neurodegenerative disorders.⁽¹⁾ To study the protein expression and localization of EFhd2 and to assess proteolytic degradation of EFhd2, specific monoclonal antibodies (MAb) are required. These should recognize murine and human EFhd2 and not EFhd1.

¹Division of Molecular Immunology, Department of Medicine III, University of Erlangen-Nürnberg, Nikolaus Fiebiger Center, Erlangen, Germany.

²Institute for Neuropathology, Department Kopfklinikum, University of Erlangen-Nürnberg, Erlangen, Germany.

³Medizinisch-Naturwissenschaftliches Forschungszentrum, Universität Tübingen, Tübingen, Germany.

To be able to stain EFhd2, we generated anti EFhd2 monoclonal antibodies that recognize murine and human EFhd2, but not EFhd1. To detect the latter specifically, we also generated specific anti EFhd1 polyclonal antibodies. We reveal that the anti EFhd2 MAb bind to the N-terminal 60 amino acids of EFhd2 and established specific staining protocols. Finally, we assessed expression of EFhd2 in PBMC of healthy humans. We conclude that EFhd2 is ~5 fold more strongly expressed in monocytes than in B cells.

Materials and Methods

Chemicals

All chemicals were purchased from Sigma-Aldrich (Deisenhofen, Germany), Merck (Darmstadt, Germany), or Roth (Karlsruhe, Germany) unless stated otherwise. Cell culture medium and supplements were obtained from Invitrogen Life Technologies (Heidelberg, Germany).

Cell lines

WEHI231 B cell lines with silenced or reconstituted EFhd2 expression were maintained as described previously.⁽¹⁷⁾ Briefly, WEHI231 B cells where the EFhd2 mRNA is silenced through stable expression of a shRNA (WEHI231.sh35)⁽⁵⁾ had been infected with a retrovirus (pMSCVneo) (-), or with pMSCVneo encoding a Myc-tagged EFhd2 (+) or EFhd2 mutants whose mRNAs lack the shRNA binding site.^(3,17) The murine B cell lines 38B9,⁽¹⁸⁾ NFS-5,⁽¹⁹⁾ WEHI231,^(20,21) CH27.LX,⁽²²⁾ and P3-X63-Ag8 (Ag8)⁽²³⁾ were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M β -ME, and 100 μ g/mL penicillin-streptomycin (R10) at 37°C, 5% CO₂, and 95% humidity.

Recombinant proteins

The EcoRI fragment from pCR2.1 containing the EFhd2 coding sequence⁽⁵⁾ was cloned into the EcoRI site of pGEX-2T (27-4801-01) (Amersham Pharmacia Biotech, Freiburg, Germany). pGEX-2T-EFhd2 was transformed into *Escherichia coli* Rosetta (Invitrogen) and expression was induced with 1 mM Isopropyl β -D-1-thiogalactopyranoside (IPTG, Peqlab, Erlangen, Germany) for 2 h at 37°C. GST and GST-EFhd2 were purified using Glutathione SepharoseTM 4B (GE Healthcare, Munich, Germany) according to the manufacturer's instructions, eluted with 100 mM GSH in 200 mM Tris/HCl (pH 8.0), 140 mM NaCl, 1 mM dithiothreitol (DTT; Sigma, St. Louis, MO), dialysed extensively against 20 mM Tris (pH 8.0), 140 mM NaCl, and 1 mM DTT, and stored at -70°C.

Generation of anti-EFhd2 MAbs

Mice (BALB/c) were purchased from Janvier (Elevage Janvier, Le Genest St. Isle, France) and maintained in cages with filter covers in an environmentally controlled room in the Nikolaus Fiebiger Center, Friedrich-Alexander-University Erlangen-Nürnberg. All animal immunization and experiments were conducted according to institutional ethical guidelines for animal handling. Three 13-week-old female BALB/c mice were immunized with 50 μ g GST-EFhd2 1:1 in complete Freund's adjuvant intraperitoneally (i.p.). Second and third immunizations were performed on days 25 and

45 i.p. with 10 μ g GST-EFhd2 mixed 1:1 with incomplete Freund's adjuvant. Titers were tested by Western blot analysis at days 35 and 58 with 1:200 diluted sera, and the mouse with the highest response was finally immunized 5 days before fusion with 10 μ g GST-EFhd2 fusion protein in PBS intravenously (i.v.). Fusion was performed as described previously.⁽²⁴⁾ After 2 weeks, clones were screened by ELISA. Briefly, microtiter plates (Microton, medium binding, Greiner, Frickenhausen, Germany) were coated either with 1 μ g/mL GST or with 2 μ g/mL GST-EFhd2 fusion protein at 4°C overnight in 15 mM Na₂CO₃/35 mM NaHCO₃, washed three times with 0.05% Tween-20 in PBS, and blocked with 2% FBS in PBS. Hybridoma supernatants were applied and bound antibodies were detected with a horseradish peroxidase (HRP) conjugated goat anti mouse Fc γ fragment specific antibody (Jackson, distributed by Dianova, Hamburg, Germany) and O-phenylenediamine with acid stop. Absorption was measured with a Spectra max 190 spectrophotometer (Molecular Devices, Ismaning, Germany) at a wavelength of 490 nm. Clones reactive with GST-EFhd2 but not GST alone were further validated by Western blot and subcloned twice by limiting dilution. Antibodies were purified on Protein G columns (GE Healthcare, Munich, Germany) according to standard methods.⁽²⁵⁾

Other antibodies and staining reagents

Rabbit anti-actin antibody was purchased from Sigma. The rabbit polyclonal anti-EFhd2 antibody has been described previously.⁽⁸⁾ A goat antibody recognizing the C-terminus of EFhd2, but not EFhd1 (our unpublished data) was purchased from Everest Biotech (Upper Heyford, Oxfordshire, United Kingdom). The antibody against murine Swiprosin-2/EFhd1 (mEFhd1) is described as follows. The mEFhd1 peptide with an additional cysteine residue, RLEVRAETDQGDPC, was coupled with Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC; Thermo Scientific, Bonn, Germany) to keyhole limpet hemocyanin (KLH). Conjugates were purified by gel filtration, New Zealand White rabbits were immunized and boosted according to standard procedures,⁽²⁵⁾ and antibody titers were tested by Western blotting. The serum was affinity purified on a mEFhd1 peptide column,⁽²⁵⁾ generated by coupling the peptide used for immunization to SulfoLink Agarose (Thermo Scientific).

Goat anti-rabbit IgG (H+L) coupled to HRP was purchased from Bio-Rad (Munich, Germany) and Fc γ fragment specific goat anti-mouse antibody coupled to HRP came from Jackson. Cy3-conjugated, Fc γ fragment specific goat anti-mouse antibodies were obtained from Jackson and anti Myc antibody 9E10 has been previously described.⁽²⁶⁾

Transfection and Western blot analysis

The murine cDNA for EFhd1 was cloned from murine kidney RNA. Total RNA was obtained, reversely transcribed for 30 min at 50°C and amplified in a single step PCR reaction⁽⁵⁾ with specific primers ACTACGCGCTGAAAGCTGC and CATCATGTCCAGCGAGG at 56°C annealing temperature for 30 cycles. The product was cloned into pCR2.1 by TA cloning (Invitrogen) and sequenced. A BamHI/EcoRV fragment was then cloned into the doxycycline inducible vector pWHE467⁽²⁷⁾ to generate pWHE467mEFhd1. The expression vector for human EFhd2 (pCMVSPORT6_hEFhd2) was obtained from imaGenes (Berlin, Germany) as sequence

FIG. 1. Specificity of anti EFhd2 MABs. **(A)** Lysates of EFhd2-silenced (-) or EFhd2Myc-reconstituted (+) WEHI231 B cells were separated by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was cut into four strips, which were blocked and incubated with primary anti-EFhd2 MABs (0.5 μ g/mL) as indicated. Blots were also probed with anti Myc MAB 9E10. An unspecific band (*) marked with the anti Myc MAB represents the loading control. Molecular mass standards (kDa) are indicated on the left. **(B1)** Lysates of EFhd2-silenced (-), EFhd2Myc-reconstituted (+) WEHI231 B cells, and total brain were separated by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was cut into strips, which were probed with anti-EFhd2 MABs as indicated at the bottom, followed by HRP-conjugated anti-mouse antibodies. Blots were developed with ECL. Then membranes were stained with anti-actin pAb, followed by HRP-conjugated anti-rabbit antibodies. All strips are from the same blot and exposure. Molecular mass standards (kDa) are indicated on the left. **(B2)** Lysates of EFhd2-silenced (-), EFhd2Myc-reconstituted (+) WEHI231 B cells, and total brain were separated by 10% SDS-PAGE and transferred to nitrocellulose. The membrane was cut into strips, which were probed with goat and rabbit anti-EFhd2 pAbs, followed by HRP-conjugated anti-goat and anti-rabbit antibodies as indicated at the bottom. Blots were developed with ECL. Molecular mass standards (kDa) are indicated on the left. **(C)** Schematic representation of the protein structure of murine EFhd2 (mEFhd2). Lysates of EFhd2-silenced (-) WEHI231 B cells or of EFhd2-silenced WEHI231 B cells reconstituted with Myc-tagged wild-type EFhd2 (EFhd2) or with deletion mutants of EFhd2 where the low complexity (LC) region was deleted (Δ LC), the proline rich region (Δ PR), EF hands 1 or 2 (Δ EF1, Δ EF2), or the coiled-coil region (Δ CC) were separated by 10% SDS-PAGE and transferred to nitrocellulose. Blots were incubated first with anti-EFhd2 MAB as indicated, followed by anti Myc antibody followed by HRP-conjugated anti-mouse antibodies. Blots were developed with ECL. Molecular mass standards are indicated on the left. EFhd2 deletion mutants were published previously.³

TABLE 1. APPLICATIONS OF ANTI-EFHD2 AND ANTI-EFHD1 ANTIBODIES

Antibody & method	E7.20.23	A4.18.18	A5.15.48	A4.15.28	Anti-EFhd1 pAb	Anti-EFhd2 pAb
Western blotting (mouse and human protein)	+	+	+	+	+	+(mouse)
Immunofluorescence fixation/permeabilization						
PFA/Triton X-100	+	+	+	+	–	–
PFA/acetone	+	+	+	+	ND	ND
Methanol	+	+	+	+	ND	ND
Immunohistochemistry						
Paraffin, PFA fixation with antigen retrieval (unpurified hybridoma supernatants; mouse tissue)	-/ ~	+	ND	ND	ND	ND
Paraffin, PFA fixation with antigen retrieval (unpurified) and immunofluorescence (mouse and human tissue)	+	+	ND	ND	ND	ND
Cryosections (mouse)	+	+	+	+	ND	~
Flow cytometry (mouse and human protein) fixation/ permeabilization	+	+	+	+	–	–
PFA/0.1% Tween-20 (37°C)						
Immunoprecipitation (mouse protein) native conditions	+	+	+(*)	+	+	+

ND, not determined; *, immunoprecipitation possible, but MAb precipitates less efficiently than the other MAbs; +, antibody works in this application; ~, antibody works less well compared to functional antibody.

verified I.M.A.G.E. clone. Then, 293 human embryonic kidney cells (293HEK) were seeded at a density of 10^5 in 6 well plates and transfected 24 h later with 3 μ g of calcium chloride precipitated plasmid according to standard procedures.⁽²⁷⁾ Transfected cells were then either left untreated or protein expression was induced with doxycycline for another 24 h.⁽²⁷⁾ Cell lysates as well as brain lysates from mice were generated in RIPA buffer (0.1% SDS, 0.25% sodium deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM Tris/HCl (pH 7.5), 2.5 mM sodium pyrophosphate, 1 mM NaF, 1 mM sodium β -glycerophosphate, 1 mM PMSF, and 1 mM sodium vanadate) and cleared by centrifugation at 10,000 g. Proteins were separated by SDS-PAGE⁽²⁸⁾ followed by semi-dry transfer onto Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), which were blocked, probed, and developed as described previously.⁽¹⁷⁾

Immunofluorescence, flow cytometry, and immunohistochemistry

Cells were attached to Teflon coated coverslips (Roth) in serum-free medium for 30 min, stimulated, washed quickly in ice-cold PBS, and fixed in 100% MeOH for 15 min at -20°C (alternatively, 4% paraformaldehyde (PFA) in PBS for 15 min on ice, followed by permeabilization with 0.1% NP-40 or Triton X-100 for 5 min at room temperature [RT]), or with Acetone for 1 min at RT). Cells were rinsed in PBS, blocked with 10% FBS/PBS (block buffer), and stained with antibodies at the indicated concentrations in block buffer. After washing in PBS, cells were stained with secondary antibodies diluted in PBS supplemented with 2% FBS, washed again in PBS, and mounted in MOWIOL (Hoechst, Frankfurt, Germany). For flow cytometry, cells were pelleted, washed with PBS, and directly analyzed in a Becton Dickinson FACSCalibur flow cytometer as described.⁽⁵⁾ For intracellular staining, cells were fixed with 4% PFA in PBS for 15 min on ice, washed with PBS, and permeabilized with 0.1% Tween-20 for 10 min at 37°C . Cells were then incubated with block buffer for 20 min, stained with primary and secondary antibodies diluted in block buffer, and analyzed in a Becton Dickinson FACSCali-

bur flow cytometer. PFA fixed tissue sections embedded in paraffin were de-paraffinized and antigen was retrieved automatically.⁽²⁹⁾ Briefly, de-paraffinized sections were heated for 30 min in a Benchmark-Stainer (Roche) in 10 mM Tris, 1 mM EDTA, and 0.05% Tween-20 at 95°C .

Staining of human peripheral blood monocyctic cells

Studies with human PBMC were performed according to the principles outlined by the Helsinki Declaration. Appropriate informed consent was obtained. Human PBMCs were isolated from blood samples of volunteers by density gradient centrifugation (Lymphoflot, Biotest, Dreieich, Germany). Living cells were resuspended in 2% FCS/PBS buffer (30 μ L/ 10^7 cells), FC receptors were blocked with human Ig (10 μ L per 30 μ L buffer from Monocyte Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany) for 10 min at 4°C and washed with 2 mL buffer. 0.5×10^6 cells were fixed with 4% PFA/PBS for 15 min at RT, washed, and permeabilized with 0.1% Tween/PBS for 10 min at 37°C . Cells were stained with or without preincubation with equimolar amounts of GST (5 μ g/mL) or GST-EFhd2 fusion protein (10 μ g/mL) for 20 min at 4°C intracellularly with 10 μ g/mL A4.18.18 or murine IgG1 κ (M9269, Sigma) as isotype control. After washing, cells were incubated with goat anti-mouse Alexa647 coupled IgG Fc γ specific antibody (115-605-071, Dianova) and washed. Samples were post-fixed with 1% PFA for 10 min at RT and washed with 0.1% Tween/PBS. Then cells were stained with anti-human FITC coupled CD19 (555412, BD Pharmingen), washed and analyzed.

Image acquisition

Images were acquired with a Leica TCS2 confocal microscope (Mannheim, Germany) with separate recording of each channel. Exposure times were calibrated with the Glow over/under function of Leica software. For EFhd2 staining, stained cells without EFhd2 expression were set as background controls and all following images were taken with same exposure times. Autofluorescence was removed automatically.

FIG. 2. Specificity of anti-EFhd2 MAbs. (A) Schematic representation of the protein structure of murine EFhd2 (mEFhd2) and the localization of the peptides used to generate anti-EFhd1 and anti-EFhd2 pAbs (red). The sequence representing the predicted low complexity region of murine (m) EFhd2, along with its homologous and paralogous sequences of mEFhd1 as well as human (h) EFhd2 and EFhd1 is depicted. Only the first ~60 amino acids are shown. (B) 293HEK cells were transfected with pCMVSPORT6 encoding human EFhd2 (hEFhd2) or the inducible vector pWHE467 encoding murine EFhd1 (mEFhd1). mEFhd1 transfected cells were either left untreated or incubated with Doxycycline for 24 h. Then, lysates were prepared and separated 5 times in parallel by 10% SDS-PAGE. Proteins were transferred to nitrocellulose, the membrane was cut into strips and the first strip was incubated with goat anti EFhd2 pAb (0.5 µg/mL) and the following strips with anti EFhd2 MAb (0.5 µg/mL), followed by HRP-conjugated anti goat and anti mouse secondary antibodies, as indicated (lower panel). Blots were developed with ECL. Then, HRP-coupled antibodies on the membrane were inactivated with NaN_3 and the membrane was stained with anti actin pAb (45 kDa) (upper panel) and anti EFhd1 pAb (middle panel) (31 kDa), followed by HRP-conjugated anti rabbit antibodies. The arrows indicate main expression products whereas the stars mark degradation products of smaller size or larger proteins with post-translational modifications. (C) Lysates of the indicated B cell lines were resolved by 10% SDS-PAGE, blotted onto nitrocellulose and stained with anti EFhd2 and anti EFhd1 antibodies. 38B9 B cells were lysed and subjected to immunoprecipitation (IP) with anti EFhd1, anti EFhd2 and normal rabbit pAbs (Ctrl.). Immunoprecipitates were resolved by 10% SDS-PAGE, blotted onto nitrocellulose and stained with anti EFhd2 and anti EFhd1 antibodies. IgH, immunoglobulin heavy chain; WCL, whole cell lysate. Molecular mass standards (kDa) are indicated on the left.

Results

To stain specifically EFhd2, we generated four anti EFhd2 IgG1κ secreting hybridomas (A4.15.28, A4.15.48, A4.18.18, E7.20.23). Antibodies were purified from hybridoma supernatants and their specificity was verified by Western blot analysis. WEHI231 B cells lacking EFhd2 expression by stable shRNA silencing (-), or WEHI231 B cells with reconstituted expression of Myc-tagged EFhd2 (+) (see Materials and Methods section) served as controls. Figure 1A demonstrates immunoreactivity of anti EFhd2 MAbs on Western blots only in WEHI231 B cell lines expressing EFhd2. *Efh2* message is expressed abundantly in the brain⁽⁵⁾ and others revealed EFhd2 protein expression in brain lysates using a commercial goat anti-EFhd2 and custom-made rabbit anti-EFhd2 antibodies.⁽³⁰⁾ Here, we confirmed protein expression of EFhd2 in murine brain with the new anti-EFhd2 MAbs (Fig. 1, B1). The commercial goat anti-EFhd2 pAb and our polyclonal rabbit anti-EFhd2 pAb⁽⁸⁾ gave similar results (Fig. 1, B2). The specificity of the anti EFhd2 MAbs was further tested by immunoprecipitation and their application in immunohistochemistry, immunocytochemistry, and intracellular flow cytometry was worked out (Table 1). To map the binding sites of the anti EFhd2 MAbs, we made use of WEHI231 cells devoid of EFhd2 expression and of WEHI231 cells expressing wild-type Myc-tagged EFhd2 or Myc-tagged EFhd2 mutants.⁽³⁾ In those mutants, the N-terminal low complexity region had been deleted (ΔLC), the proline rich stretch (ΔPR), EF hands 1 and 2 (ΔEF1, ΔEF2), or the C-terminal coiled-coil domain (ACC)⁽³⁾ (Fig. 1C, schematic). This experiment revealed that anti-EFhd2 MAbs did not recognize EFhd2 lacking the N-terminal low complexity region whereas the anti-Myc antibody did (Fig. 1C). Thus, the anti-EFhd2 MAbs recognize an epitope within the N-terminal low complexity region.

EFhd2 is highly conserved among species, especially among mouse, rat, and man⁽⁵⁾ and has a close homologue, Swiprosin-2/EFhd1 (EFhd1) with 69.7% sequence identity and the same predicted protein structure.⁽¹⁾ EFhd1 differs from EFhd2 significantly only in some parts of the N-terminal region before the EF hands⁽¹⁾ (Fig. 2A). To specifically detect mEFhd1, we generated a polyclonal rabbit antibody directed against a unique peptide of the N-terminus of mEFhd1 (Fig. 2A). To assess whether the anti-EFhd2 MAbs also recognize human EFhd2 (hEFhd2), and to exclude that they recognize murine EFhd1 (mEFhd1), we left 293HEK cells untransfected, transfected them with a vector encoding hEFhd2 or with a doxycycline inducible vector encoding mEFhd1. Lysates were then analyzed by Western blotting. Figure 2B reveals that the rabbit anti-EFhd1 pAb recognizes ectopic mEFhd1 in 293HEK cells but not ectopically expressed hEFhd2. In contrast, antibodies from all four hybridomas react on Western blot analysis with hEFhd2. Remarkably, they do not recognize the related mEFhd1. Ectopic expression of hEFhd2 and mEFhd1 revealed differentially migrating bands. Since this pattern was obtained only after expression from cDNA constructs, the different bands represent only transfected protein. The arrows indicate the expected main bands, and the asterisks indicate possibly different post-translational modifications or degradation products of the over-expressed proteins (Fig. 2B).

Next we confirmed the specificity of anti-mEFhd1/EFhd2 antibodies. We first analyzed expression of EFhd1 and EFhd2

in transformed murine B cell lines representing originally various differentiation states, namely pro B cells (38B9), pre B cells (NFS5), activated immature and mature B cells (WEHI231, CH27.LX), and plasma cells (Ag8). Whereas only 38B9 cells express EFhd1 on the protein level, EFhd2 is expressed in all B cell lines (Fig. 2C). To exclude that the anti-mEFhd1 antibody recognizes mEFhd2 and vice versa, we precipitated either EFhd2 or EFhd1 from 38B9 and WEHI231 cell lysates and probed the precipitates reciprocally, either with anti-EFhd1 or anti-EFhd2 pAbs (Fig. 2C). This experiment unequivocally demonstrates that anti-EFhd1 and anti-EFhd2 pAbs only recognize EFhd1 and EFhd2, respectively. Other features of anti-EFhd1/2 pAbs can be gleaned from Table 1.

FIG. 3. EFhd2 staining in WEHI231 cells. (A) EFhd2-silenced (-) or EFhd2Myc-reconstituted (+) WEHI231 B cells were attached to glass slides, fixed with PFA and permeabilized. Fixed cells were then stained with anti EFhd2 MAbs as indicated, followed by Cy3-conjugated rabbit anti mouse antibody. After mounting, cells were examined by confocal microscopy (1 Z layer, 8 averaged images). Scale bar, 10 μm. (B) EFhd2Myc-reconstituted WEHI231 B cells were attached to glass slides, fixed with PFA and permeabilized. Fixed cells were then stained with anti EFhd2, anti Calnexin or anti Calreticulin antibodies. After mounting, cells were examined by confocal microscopy (1 Z layer, 6 averaged images). Scale bar, 10 μm.

Previously we have shown that an EFhd2-EGFP fusion protein labels the plasma membrane and intracellular structures.⁽⁵⁾ To determine the subcellular localization of EFhd2 in B cells using antibodies, we stained WEHI231.shEFhd2 (-) and WEHI231.EFhd2 (+) cells for EFhd2 (Fig. 3A). EFhd2 localizes

at the plasma membrane but also to a great extent in intracellular, putatively vesicular structures (Fig. 3A). To exclude unspecific binding of the MAbs to the endoplasmatic reticulum (ER), we stained WEHI231.EFhd2 cells with antibodies recognizing the ER chaperones Calnexin and Calreticulin,

FIG. 4. Flow cytometric EFhd2 staining in primary murine and human cells. (A) Primary splenic B cells were stimulated with lipopolysaccharide and infected with a retrovirus encoding an IRES-GFP cassette or EFhd2Myc followed by an IRES-GFP cassette. 48 h later, cells were stained with anti EFhd2 MAb A4.18.18, followed by secondary Cy3-conjugated mouse Fcγ [specific Ab (FL2)]. Similar results were obtained with the other MAb. (B) Human PBMC were fixed, permeabilized and stained for CD19 and EFhd2 (MAb A4.18.18) as indicated. A murine IgG1κ antibody served as isotype control. Cells were analyzed in monocyte and lymphocyte gates defined by forward scatter (FSC) and side scatter (SSC). Numbers indicate % cells in the gates. (C) Prior to anti EFhd2 staining of human PBMC, anti EFhd2 MAb A4.18.18 was incubated with a threefold molar amount of either GST or GST-EFhd2 fusion protein for 15 min. Shown are human B cells. Representative of two experiments. Numbers indicate % cells in the gates.

revealing a different staining pattern from that with the anti-EFhd2 MAbs (Fig. 3B). We conclude that EFhd2 does not, or at least not to a significant extent, localize to the ER. Similarly, EFhd2 was stained outside of the nucleus at the membrane and in perinuclear, vesicular structures (Fig. 3B). This is in full compatibility with subcellular fractionation assays where we never detected a nuclear localization of EFhd2 (data not shown).

Having established the specificity and human species reactivity of the anti-EFhd2 MAbs, we wished to assess the suitability of anti-EFhd2 MAbs in flow cytometric staining of PBMC. First we stained murine primary B cells forced to express GFP alone or in combination with EFhd2 through retroviral infection.⁽⁵⁾ Cells expressing GFP alone were set to zero on the Y-axis (Fig. 4A, left plot). With these settings, we showed that the anti-EFhd2 MAb specifically and dose dependently recognize EFhd2 in EFhd2 over-expressing B cells (Fig. 4A, right plot). Thus, this method is suitable to quantify EFhd2 expression in a linear manner over two log scales by flow cytometry.

Next we analyzed EFhd2 expression in human PBMC by flow cytometry (Fig. 4B). Freshly isolated human PBMC were stained with an isotype-matched antibody (IgG1 κ) or anti-EFhd2 antibody A4.18.18. We gated on monocytes as well as B cells, which revealed EFhd2 expression in human monocytes and B cells, as expected. However, human monocytes express approximately five times more EFhd2 than human B cells (Fig. 4B). To control once more the specificity of this staining procedure, we pre-incubated the anti-EFhd2 MAb either with GST alone or with the GST-EFhd2 fusion protein (Fig. 4C), demonstrating a complete block of the specific EFhd2 staining by GST-EFhd2.

Discussion

Here we describe the generation and application of specific anti EFhd2 MAbs. The four anti-EFhd2 MAbs we chose do recognize murine as well as human EFhd2. Moreover, they do not recognize murine EFhd1, which differs from EFhd2 mostly in the N-terminal part. Apparently, the N-terminal part of EFhd2 that discriminates this protein from EFhd1 contains immunodominant epitopes, of which we also have made use of here and previously⁽⁸⁾ by generating specific polyclonal antibodies. In line with our results obtained with an EFhd2-GFP fusion protein,⁽⁵⁾ we reveal two major EFhd2 pools in B cells, one at the plasma membrane and an intracellular pool that does not represent the endoplasmic reticulum. Identification of the intracellular structures stained by anti-EFhd2 antibodies remains a task for the future. EFhd2 has been reported to be expressed in CD4 positive T helper cells and cytotoxic cells, such as CD8 T cells and NK cells, but also in antigen-presenting cells, such as B cells and macrophages (see review⁽¹⁾). In addition to this point, we reveal a five times higher expression of EFhd2 protein in human monocytes than in human B cells by flow cytometry.

Since we established this staining procedure, it will be possible in the future to assess the detailed expression of EFhd2 in PBMC subclasses of healthy donors and of those suffering from inflammatory or neurodegenerative diseases. By combining our antibodies that recognize the N-terminal part of the protein with those recognizing the C-terminal part, such as commercial ones (see Materials and Methods), it will

even be possible to define EFhd2 cleavage patterns.⁽¹⁶⁾ Taken together, EFhd2 monoclonal antibodies are valuable to assess the real subcellular localization and expression of EFhd2 in primary cells and tissues, in the healthy and diseased. EFhd2 is highly expressed in the brain and putatively involved in the pathophysiology of Alzheimer's disease.^(1,31) Thus, future studies should also be directed to analyze EFhd2 expression in normal and diseased human brain. Therefore it is of great advantage to know that all anti-EFhd2 antibodies we generated do not recognize EFhd1 and vice versa. Hence, EFhd1 and EFhd2 protein expression in tissue can be reliably assessed in the future.

Acknowledgments

We thank Verena Schmitt for excellent technical assistance. This work was supported by grants from the German Science Foundation (Deutsche Forschungsgemeinschaft; Mi832/2-2 to D.M.) and the Interdisciplinary Clinical Research Center Erlangen (IZKF Erlangen; grant E8 to D.M.).

Author Disclosure Statement

The authors have no financial interests to disclose.

References

1. Dütting S, Brachs S, and Mielenz D: Fraternal twins: Swiprosin-1/EFhd2 and Swiprosin-2/EFhd1, two homologous EF-hand containing calcium binding adaptor proteins with distinct functions. *Cell Commun Signal* 2011;9:2.
2. Tominaga M, Kurihara H, Honda S, Amakawa G, Sakai T, and Tomooka Y: Molecular characterization of mitocalcin, a novel mitochondrial Ca²⁺-binding protein with EF-hand and coiled-coil domains. *J Neurochem* 2006;96:292–304.
3. Hagen S, Brachs S, Kroczeck C, Fumrohr BG, Lang C, and Mielenz D: The B cell receptor-induced calcium flux involves a calcium mediated positive feedback loop. *Cell Calcium* 2012;51:411–417.
4. Ferrer-Acosta A, Rodriguez-Cruz EN, Vaquer-Alicea AC, and Vega IE: Functional and structural analysis of the conserved EFhd2 protein. *Protein Pept Lett* 2012;20: 5730583.
5. Avramidou A, Kroczeck C, Lang L, Schuh W, Jack HM, and Mielenz D: The novel adaptor protein Swiprosin-1 enhances BCR signals and contributes to BCR-induced apoptosis. *Cell Death Differ* 2007;14:1936–1947.
6. Martins-de-Souza D, Gattaz WF, Schmitt A, Rewerts C, Maccarrone G, Dias-Neto E, and Turck CW: Prefrontal cortex shotgun proteome analysis reveals altered calcium homeostasis and immune system imbalance in schizophrenia. *Eur Arch Psychiatry Clin Neurosci* 2009;259:151–163.
7. Alkelai A, Lupoli S, Greenbaum L, Giegling I, Kohn Y, Sarner-Kanyas K, Ben-Asher E, Lancet D, Rujescu D, Macciardi F, and Lerer B: Identification of new schizophrenia susceptibility loci in an ethnically homogeneous, family-based, Arab-Israeli sample. *Faseb J* 2011;25:4011–4023.
8. Mielenz D, Vettermann C, Hampel M, Lang C, Avramidou A, Karas M, and Jack HM: Lipid rafts associate with intracellular B cell receptors and exhibit a B cell stage-specific protein composition. *J Immunol* 2005;174:3508–3517.
9. Zhai J, Strom AL, Kilty R, Venkatakrishnan P, White J, Everson WV, Smart EJ, and Zhu H: Proteomic characterization of lipid raft proteins in amyotrophic lateral sclerosis mouse spinal cord. *FEBS J* 2009;276:3308–3323.

10. Perry JJ, Shin DS, and Tainer JA: Amyotrophic lateral sclerosis. *Adv Exp Med Biol* 201;685:9–20.
11. Mutez E, Larvor L, Lepretre F, Mouroux V, Hamalek D, Kerckaert JP, Perez-Tur J, Waucquier N, Vanbesien-Mailliot C, Duflot A, Devos D, Defebvre L, Kreisler A, Frigard B, Destee A, and Chartier-Harlin MC: Transcriptional profile of Parkinson blood mononuclear cells with LRRK2 mutation. *Neurobiol Aging* 2011;32:1839–1848.
12. Ullrich C, Mlekusch R, Kuschnig A, Marksteiner J, and Humpel C: Ubiquitin enzymes, ubiquitin and proteasome activity in blood mononuclear cells of MCI, Alzheimer and Parkinson patients. *Curr Alzheimer Res* 2012;7:549–555.
13. Hornbruch-Freitag C, Griemert B, Buttgerit D, and Renkawitz-Pohl R: Swiprosin-1/EFhd2 accumulates at the prefusion complex stage during myoblast fusion. *J. Cell Sci* 2011;124:3266–3278.
14. Xu Y, Liu W, Shen H, Yan J, Yang E, and Wang H: Recombinant Mycobacterium bovis BCG expressing chimaeric protein of Ag85B and ESAT-6 enhances immunostimulatory activity of human macrophages. *Microbes Infect* 2010;12:683–689.
15. Dotzlaw H, Schulz M, Eggert M, and Neeck G: A pattern of protein expression in peripheral blood mononuclear cells distinguishes rheumatoid arthritis patients from healthy individuals. *Biochim Biophys Acta* 2004;1696:121–129.
16. Schulz M, Dotzlaw H, Mikkat S, Eggert M, and Neeck G: Proteomic analysis of peripheral blood mononuclear cells: selective protein processing observed in patients with rheumatoid arthritis. *J Proteome Res* 2007;6:3752–3759.
17. Kroczeck C, Lang C, Brachs S, Grohmann M, Dutting S, Schweizer A, Nitschke L, Feller SM, Jack HM, and Mielenz D: Swiprosin-1/EFhd2 controls B cell receptor signaling through the assembly of the B cell receptor, Syk, and phospholipase C gamma2 in membrane rafts. *J Immunol* 2010;184:3665–3676.
18. Alt F, Rosenberg N, Lewis S, Thomas E, and Baltimore D: Organization and reorganization of immunoglobulin genes in A-MULV-transformed cells: rearrangement of heavy but not light chain genes. *Cell* 1981;27:381–390.
19. Kudo A, Thalmann P, Sakaguchi N, Davidson WF, Pierce JH, Kearney JF, Reth M, Rolink A, and Melchers F: The expression of the mouse VpreB/lambda 5 locus in transformed cell lines and tumors of the B lineage differentiation pathway. *Int Immunol* 1992;4:831–840.
20. Boyd AW, and Schrader JW: The regulation of growth and differentiation of a murine B cell lymphoma. II. The inhibition of WEHI 231 by anti-immunoglobulin antibodies. *J Immunol* 1981;126:2466–2469.
21. Spillmann FJ, and Wabl M: Endogenous expression of activation-induced cytidine deaminase in cell line WEHI-231. *J Immunol* 173:1858–1867.
22. Bishop GA, and Haughton G: Use of the CH lymphomas as models of murine B cell differentiation. *Immunol Res* 2004;5:263–270.
23. Humphrey RL, Trivedi SM, and Frondoza CG: Studies with murine LPC-1 plasmacytoma using [6-14C]arginine. *Cell Tissue Kinet* 1979;12:71–80.
24. Mielenz D, Ruschel A, Vettermann C, and Jack HM: Immunoglobulin mu heavy chains do not mediate tyrosine phosphorylation of Ig alpha from the ER-cis-Golgi. *J Immunol* 2003;171:3091–3101.
25. Harlow E, and Lane D: *Antibodies—A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988.
26. Evan GI, Lewis GK, Ramsay G, and Bishop JM: Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol Cell Biol* 1985;5:3610–3616.
27. Krueger C, Danke C, Pfeleiderer K, Schuh W, Jack HM, Lochner S, Gmeiner P, Hillen W, and Berens C: A gene regulation system with four distinct expression levels. *J Gene Med* 2006;8:1037–1047.
28. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680–685.
29. Buslei R, Holsken A, Hofmann B, Kreutzer J, Siebzehnruhl F, Hans V, Oppel F, Buchfelder M, Fahlbusch R, and Blumcke I: Nuclear beta-catenin accumulation associates with epithelial morphogenesis in craniopharyngiomas. *Acta Neuropathol* 2007;113:585–590.
30. Vega IE, Traverso EE, Ferrer-Acosta Y, Matos E, Colon M, Gonzalez J, Dickson D, Hutton M, Lewis J, and Yen SH: A novel calcium-binding protein is associated with tau proteins in tauopathy. *J Neurochem* 2008;106:96–106.
31. Borger E, Aitken L, Muirhead KE, Allen ZE, Ainge JA, Conway SJ, and Gunn-Moore FJ: Mitochondrial beta-amyloid in Alzheimer's disease. *Biochem Soc Trans* 2011; 39:868–873.

Address correspondence to:

Dr. Dirk Mielenz

Division of Molecular Immunology

Department of Internal Medicine III

University of Erlangen-Nürnberg

Nikolaus Fiebiger Center

Glückstrasse 6

91054 Erlangen

Germany

E-mail: dmielenz@molmed.uni-erlangen.de

Received: February 21, 2013

Accepted: March 28, 2013