Generating a Battery of Monoclonal Antibodies Against Native Green Fluorescent Protein for Immunostaining, FACS, IP, and ChIP Using a Unique Adjuvant

Philip Sanchez, Karla J. Daniels, Yang-Nim Park, and David R. Soll

Using a unique, nontoxic adjuvant compound of poly(I:C) and anti-CD40 MAb, a battery of eight mouse monoclonal antibodies was generated against native green fluorescent protein. All were effective to varying degrees for immunostaining paraformaldehyde-fixed cells, six for staining sections of paraffin-embedded tissue, all to varying degrees in fluorescent-activated cell sorting, five for immunoprecipitation, and seven for chromatin immunoprecipitation. None worked in denaturing Western blots since the target was the native GFP protein. Both the hybridomas and antibodies are available at cost through DSHB, a non-profit National Resource created by the National Institutes of Health.

Introduction

FLUORESCENT EPITOPE TAGS are valuable for assessing the localization of proteins in cells and tissues. In addition, through the use of antibodies against the tags, they can be used to identify the proteins and, in the case of transcription factors, the DNA sequences they interact with, by immunoprecipitation (IP) and chromatin immunoprecipitation (ChIP), respectively. Green fluorescent protein (GFP) has proven to be one of these effective tags. It is fluorescent and can also be identified by antibodies. GFP was discovered by Shimomura and colleagues⁽¹⁾ in the jellyfish *Aequorea victoria*. It is comprised of 11 beta-sheets forming a barrel, with an alpha-helix through the center.⁽²⁾ The chromophore consists of three amino acids in the alpha-helix. GFP derivatives, such as eGFP, have been developed with mutations in the chromophore to increase fluorescence.⁽³⁾ Other GFP mutants have been produced that cause changes in color such as blue fluorescent protein (BFP), cyan fluorescent protein (CFP), and yellow fluorescent protein (YFP). These derivatives cause changes in the excitation/emission spectra.⁽⁴⁾ GFP that is tagged to a protein at a site that does not interfere with function allows visualization of the protein in live and, in some cases, fixed preparations. Antibodies against GFP have also proven useful in IP and ChIP.^(5,6) Antibodies against GFP can be generated against the denatured protein, a peptide, or the wild-type native protein. In the latter case, the antibodies may prove more effective in IP and ChIP, in which the targets are native proteins.

Because of innumerable requests by clients of the Developmental Studies Hybridoma Bank (DSHB), a non-profit

National Resource created by NIH, for hybridomas that produce antibodies against GFP that are effective in IP and ChIP, we have generated a battery of eight mouse hybridomas that produce anti-native GFP monoclonal antibodies (MAbs) using a unique, less toxic adjuvant, formerly used in vaccine development.⁽⁷⁾ Characterization of these eight MAbs is presented here. All eight proved effective for immunostaining fixed cell preparations, six for staining sections of paraffin-embedded tissue, all for fluorescence-activated cell sorting (FACS), five for IP and seven for ChIP. Two hybridomas, 12A6 and 8H11, produced MAbs that were the best all-around performers. None were effective for Western blotting, most likely because they were generated against native protein. Both the MAbs and hybridomas are now available to basic researchers at cost through the DSHB.

Materials and Methods

Mice and immunization

Two female BALB/c mice (8 weeks old) were immunized by intraperitoneal injection of 20 µg recombinant native GFP (Brighter Ideas, North Brunswick, NJ) combined with 50 µg of HMW VacciGrade (poly(I:C); InvivoGen, San Diego, CA) and 50 µg of anti-CD40 monoclonal antibody (BioXcell, West Lebanon, NH) as the adjuvant,^(7,8) in a total volume of 200 µL in phosphate buffer solution (PBS). Mice were boosted 2 weeks after initial immunization by injection of 10 µg of recombinant native GFP and 15 µg of poly(I:C) in 200 µL of phosphate buffered saline (PBS; Gibco, Grand Island, NY). Mice were bled 3 weeks after the boost, and immune sera tested for anti-GFP reactivity by immunofluorescence. Two

months after the first boost, and 3 days prior to fusion, the mouse with the highest titer was given a second intravascular injection with 5 μ g of native GFP and 10 μ g of poly(I:C) in 200 μ L of PBS.

Hybridoma fusion

Immunized spleen cells and Sp2/0-Ag14 myeloma cells (CRL-1581, ATCC, Manassas, VA)⁽⁹⁾ were washed, passed through a 70 μ m filter, and resuspended in RPMI 1640 medium (Life Technologies, Carlsbad, CA) containing 100 μ M hypoxanthine and 16 μ M thymidine (HT Media Supplement Hybri-Max; Sigma-Aldrich, St. Louis, MO). Cells were mixed at a ratio of five nucleated splenocytes to one myeloma cell (5:1) and pelleted. The cell pellet was dispersed by gently tapping the tube and warming to 37°C for one min; then 0.5 mL of pre-warmed 50% (v/v) PEG-RPMI 1640 medium was gently added. Cells were gently mixed by tapping for 2 additional min at 37°C, and 15 mL of warm serum-free RPMI 1640 medium was added drop-wise over the next 90 s. Cells were incubated without agitation for 8 min at room temperature, placed in a 37°C water bath for 2 min, and pelleted by centrifugation at 200 g for 5 min. The supernatant was removed and the pellet dispersed by gently tapping the tube. Cells were gently dispersed by pipetting into 30 mL of the hybridoma culture medium RPMI 1640 + 15% fetal bovine serum (FBS; Hyclone, Logan, Utah) + 20% medium conditioned by giant cell tumor (TIB-223; ATCC, Manassas, VA)⁽¹⁰⁾ and 1x HT-Hybri-Max. Large cell clumps were allowed to settle and the single suspended cells were transferred to T75 flasks. This process was repeated until all cells were dispersed and transferred. Cells were incubated overnight at 37°C and 100 μ L were added to each well in 96-well tissue culture plates. Hybridomas were selected by adding 100 μ L of 2x hypoxanthine-aminopterin-thymidine medium to each well (HAT; Sigma-Aldrich). When cells grew to 25% confluency, supernatants were tested for mouse IgG production by ELISA (Bethyl Laboratories, Montgomery, TX). Positive hybridomas were expanded and supernatants retested for GFP reactivity by immunofluorescence. Hybridomas producing anti-GFP-specific antibodies were further expanded, then subcloned by fluorescence-activated cell sorting (FACS) using propidium iodide (Life Technologies) staining for viability. Antibody isotypes were determined from culture supernatants using the Mouse Monoclonal Antibody Isotyping Test Kit (AbDSerotec, Raleigh, NC) according to the manufacturer's protocols.

Screen for anti-GFP immunoreactivity

A431 cells expressing GFP-tagged E-cadherin (generously provided by Dr. Christopher Stipp, University of Iowa) were grown in Dulbecco's Modified Eagles Medium (DMEM; Life Technologies) supplemented with 10% FBS and 200 μ g/mL of geneticin (G418; Sigma-Aldrich). Cells were released from the culture flask by treating them for 30 min with 5 mM EDTA in Hank's Balanced Salt Solution without cations (HBSS; Life Technologies) at 37°C, 0.25% Trypsin-EDTA solution (Sigma-Aldrich) added at a 1:4 dilution, and the cells were then incubated for 2 additional min. Cells were then washed and resuspended in DMEM medium, then replated in sterile 12-well Teflon-coated microscope slides (Tekdon, Myakka City, FL) at 2.5×10^4 cells per well. Cells were in-

cubated for up to 48 h at 37°C to allow cell attachment and spreading. Slides were rinsed briefly in HBSS, again in PBS, and then fixed in 4% paraformaldehyde (PF; Electron Microscopy Sciences, Hatfield, PA) in PBS for 10 min at room temperature. Slides were washed five times in PBS and then blocked in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% Triton X-100 (Sigma-Aldrich) for 15 min at room temperature. Slides were washed five times with PBS and 25 μ L of test antibody (supernatant, neat or purified, 1.25 μ g) were added to each well. Slides were incubated for 1 h at 37°C, washed five times with PBS, blocked with 1% normal donkey serum (Sigma) for 30 min at room temperature, and washed five times with PBS. Twenty-five μ L of secondary Alexa Fluor 546 donkey anti-mouse IgG antibody (Life Technologies) containing 5 μ g/mL of antibody was added to each well, and the preparations were incubated for 30 min at room temperature. Slides were washed five times with PBS, and a coverslip was mounted with Vectashield (Vector Laboratories, Burlingame, CA). Stained cells were imaged with a Nikon TE2000 inverted fluorescence microscope (Nikon Instruments, Melville, NY) and images captured with an attached Canon EOS Rebel T3i digital SLR camera (Canon, Melville, NY). As a negative control, MCF-7 breast adenocarcinoma cells lacking GFP fusion protein (HT22D; ATCC) were cultured, stained, and imaged as above.

Paraformaldehyde-fixed paraffin-embedded tissue

Forty-eight h post-fertilization embryos of transgenic zebrafish (generously provided by Dr. Diane Slusarski, University of Iowa) were fixed for 2 h at room temperature with 4% PF in PBS. The embryos expressed GFP driven by the *HuC* promoter, resulting in fluorescent neurons.⁽¹¹⁾ Following fixation, the embryos were washed with PBS and placed in a Paraffin Tissue Processor 1530 (RMC, Jefferson, OH) for embedding. Paraffin sections (7 μ m) were placed on slides, dried, deparaffinized, and rehydrated in a Diversified Stainer DRS-601 (Sakura, Torrance, CA). Sections were blocked with 10% normal goat serum (Sigma-Aldrich) in PBS prior to staining with 1 μ g of anti-GFP antibody (either culture supernatant or purified) at 37°C for 1 h. After extensive PBS washing, preparations were incubated in Alexa Fluor 647-conjugated goat anti-mouse IgG (Life Technologies) at 37°C for 45 min. Sections were rinsed, mounted with Vectashield (Vector Labs), and imaged as described above.

FACS

A431 cells expressing an E-cadherin-GFP fusion protein were released from culture flasks by incubation in 5 mM EDTA + 0.1% azide in HBSS at 37°C for one h. Cells were washed in cold FACS buffer (PBS + 0.1% FBS + 0.1% sodium azide), then pelleted at 300 g for 5 min. Cells were fixed and permeabilized using the BD Cytotfix/Cytoperm Kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. Aliquots of cells ($\sim 2 \times 10^5$) were resuspended with purified anti-GFP antibody diluted in BD Perm/Wash buffer, inverted to mix well, and incubated on ice for 30 min. Cells were washed three times with BD Perm/Wash buffer and resuspended in a solution containing 5 μ g/mL of Alexa Fluor 647 goat anti-mouse IgG secondary antibody in BD Perm/Wash buffer. Cells were incubated for 15 min on ice,

washed three times in BD Perm/Wash buffer, and resuspended in FACS buffer before analysis using a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using CellQuest Pro software (BD Biosciences).

Dot blot

Purified native GFP (0.25 µg) and recombinant maltose binding protein (MBP, 0.5 µg; ProSpec Proteins, East Brunswick, NJ) were spotted onto dry nitrocellulose membranes and allowed to dry. Membranes were blocked with 5% dry non-fat milk in 20 mM Tris-HCl (pH 7.4), supplemented with 150 mM NaCl and 0.1% Tween-20 (TBS-T), for one h at room temperature and then washed once in TBS-T. Tissue culture supernatant (1:10) or purified 1 µg/mL anti-GFP antibody or anti-MBP (clone P2F1; Developmental Studies Hybridoma Bank, University of Iowa) was dispersed over the membranes and incubated on a rocker overnight at 4°C. The membranes were then washed five times with TBS-T. Secondary IR680-conjugated goat anti-mouse IgG (Li-Cor Biosciences, Lincoln, NE) was added at a 1:5000 dilution over the membrane and incubated in the dark on a rocker for 30 min at room temperature. The membrane was washed five times and scanned in an Odyssey scanner (Li-Cor Biosciences).

Immunoprecipitation

White *a/a* cells of the infectious yeast *Candida albicans* strain P37005 served as the control; cells of strain P37005-*TETp-WH11-GFP*, in which the cytoplasmic white cell-specific gene *WH11*,⁽¹²⁾ fused in frame with the *eGFP* gene, was under the control of the tetracycline (doxycycline)-inducible promoter *TETp*,⁽¹³⁾ served as the test protein. The cells were grown in suspension at room temperature to $OD_{600} = 1.0$ in synthetic defined growth medium (2% glucose and 0.7% yeast nitrogen base with ammonium sulfate and amino acids) in the presence of 30 µg/mL of the inducer doxycycline, pelleted, and resuspended in 200 µL of lysis buffer (20 mM HEPES [pH 7.2]; 120 mM NaCl; 1 mM EDTA; 5% glycerol; 0.2% Triton X-100; protease inhibitor cocktail, Sigma-Aldrich). RNase- and DNase-free zirconium beads (Research Products International, Mt. Prospect, IL) were added to the cell suspension and the cells homogenized with a bead beater. WH11-GFP protein from the cell lysate was immunoprecipitated using Dynabeads Protein G (Life Technologies) bound to the test antibodies. An aliquot containing 8 µg of anti-GFP antibody was used for each IP reaction. The eluted precipitates were then analyzed in Western blots. The WH11-GFP on the blot was detected with anti-GFP monoclonal antibody cocktail (clones 7.1 and 13.1; Roche NimbleGen, Madison, WI). An Odyssey dye IR800-conjugated goat anti-mouse antibody was used as secondary antibody (Li-Cor Biosciences). Odyssey scanner and software were used to detect protein bands on the Western blots.

Chromatin immunoprecipitation

ChIP was performed on a derivative of *C. albicans*, strain SC5314, engineered to express the untagged transcription factor Bcr1 under the regulation of the tetracycline (doxycycline)-inducible promoter *TETp* (*TETp-BCR1*)⁽¹³⁾ and a test strain expressing eGFP-tagged Bcr1 under the regulation of *TETp* (*TETp-BCR1-GFP*).⁽¹⁴⁾ Cells were grown to $OD_{600} = 1.0$ in

growth medium. ChIP was performed as described by Bormann and colleagues⁽¹⁵⁾ for *Saccharomyces cerevisiae* with minor modifications.⁽¹⁶⁾ Five to 10 µg of anti-GFP antibody, as either supernatant or purified antibody, was used for each ChIP reaction. To detect Bcr1-eGFP binding to the promoter regions of two of the Bcr1 target genes, *TCC1* and *PGA31*, the harvested 200–400 bp DNA fragments from ChIP were used for the polymerase chain reaction (PCR) with primer pairs, as listed in Supplementary Table 1. The primer regions for PCR were chosen based on the ChIP-chip data by Nobile and colleagues⁽¹⁷⁾ for the promoters of the Bcr1 target genes. Since the exact DNA size (bp) of the binding regions of Bcr1 on the promoters is unknown and the harvested DNA fragments from ChIP were 200–400 bp in size, primer pairs for the peak binding regions of Bcr1 on the promoters of the genes⁽¹⁷⁾ and primer pairs partially overlapping with the peak binding regions of Bcr1 were used to ensure that the Bcr1 binding region was included in the 200 bp PCR products. *C. albicans* genomic DNA was used as a positive control template for PCR. The ChIP-PCR experiment was performed three times with independently grown cell cultures.

Results

Immunization and hybridoma generation

To generate MAbs against green fluorescent protein (GFP), mice were immunized against recombinant native (non-denatured) wild-type GFP using a novel vaccine adjuvant. The adjuvant was composed of the water-soluble constituents poly(I:C) and anti-CD40 antibody,⁽⁷⁾ obviating the need for the mineral oil emulsifier component of Complete Freund's Adjuvant (CFA). The mice were boosted 2 weeks later. Three days prior to fusion and 8 weeks after the first boost, the final boost was injected. The entire immunization protocol was completed in 10 weeks. B cells from the immune spleen cells were then fused to Sp2/0-Ag14 myeloma cells.^(9,18) A total of 444 wells were screened showing cell expansion after HAT selection. Of these, 110 tested positively for IgG production. Eight were expanded and cloned and their antibodies characterized for performance. The eight anti-GFP antibody clones and their immunoglobulin isotypes (in parentheses) are 12E6 (IgG1), 12A6 (IgG1), 8H11 (IgG1), 7F11 (IgG3), 4D5 (IgG1), 4C9 (IgG1), 2G6 (IgG1), and 1G9 (IgG1).

Indirect immunofluorescent staining of E-cadherin-GFP-expressing cells

Wild-type A431 human epidermoid carcinoma cells transfected with a gene encoding an E-cadherin-GFP fusion protein⁽¹⁸⁾ were plated in the wells of a 12-well glass slide and cultured 24–48 h to allow the E-cadherin-GFP to localize at cell-cell contacts. The cells were then fixed, permeabilized, and incubated with each of the eight anti-GFP MAbs and treated. Each antibody stained the intercellular junctions with varying efficiency (Fig. 1). MAbs 12E6, 12A6, 8H11, 7F11, 1G9, and 2G6 resulted in the most effective staining. This was true in repeat experiments (data not shown). In addition to intercellular functions, the MAbs also stained perinuclear particles in select cells (data not shown), a pattern observed with E-cadherin staining in other cell types.^(19–21) In each instance, the detected signal pattern of anti-GFP antibody

FIG. 1. Immunofluorescent staining of E-cadherin-GFP-expressing A431 human cells by MAbs. A431 human epidermal carcinoma cells expressing E-cadherin-GFP were fixed with paraformaldehyde, treated with each of the eight anti-GFP MAbs, and stained with Alexa Fluor 546 donkey anti-mouse IgG antibody. Each preparation was analyzed for GFP fluorescence and secondary antibody fluorescence. For comparison, all settings of the confocal microscope were the same for the eight stained preparations. Controls lacking a MAb did not stain. Scale bar, 50 microns.

staining (Fig. 1, right panels) overlapped GFP fluorescence (Fig. 1, left panels), indicating that the eight MAbs specifically stained GFP.

Immunofluorescent staining in paraffin-embedded sections

Paraformaldehyde-fixed transgenic zebrafish embryos were embedded in paraffin, sectioned, and stained. These transgenic embryos selectively expressed *GFP*, under the regulation of the *HUC* promoter, in neurons.⁽¹³⁾ All preparations exhibited GFP fluorescence in dorsal neural tissue, but only six of the eight MAbs (12E6, 12A6, 8H11, 7F11,

2G6 and 1G9) stained the preparations (Fig. 2). Immunostaining, when it occurred, was superimposable on the patterns of green fluorescent protein fluorescence. These results demonstrate that paraformaldehyde fixation did not affect antibody-GFP interactions for at least six of the eight anti-GFP monoclonal antibodies.

FACS

The MAbs were tested for their ability to recognize A431 cells expressing the E-cadherin-GFP fusion protein by FACS. The transfected A431 human epidermoid carcinoma cell line contained E-cadherin tagged with GFP at the carboxyl-

FIG. 2. Immunofluorescent staining of paraformaldehyde-fixed paraffin embedded tissue sections by MAbs. Forty-eight-hour transgenic zebra fish embryos expressing GFP in neurons were sectioned, treated with MAbs, and stained with Alexa Fluor647 goat anti-mouse IgG. Each preparation was analyzed for GFP fluorescence in the left panel and secondary antibody fluorescence. Controls lacking a MAb did not stain. Scale bar, 50 microns.

FIG. 3. Fluorescent activated cell sorting by MAbs. E-cadherin-GFP-expressing cells were fixed, permeabilized, treated with the anti-GFP MAbs, and stained with Alexa Fluor 647 goat anti-mouse secondary antibody. In each, the distribution of untreated cell preparation is presented in black and the MAb-treated preparation in red. The Y-axis reflects the number of cells (frequency) and the X-axis the level of fluorescence (intensity).

terminus.⁽²²⁾ Therefore, the GFP tag was located within the cell, requiring a permeable membrane prior to staining with anti-GFP antibodies. When excited by 488 nm laser, unstained A431 cells emitted in the GFP channel (530/30 band path). When the A431 cells expressing E-cadherin-GFP were sequentially excited by a 647 nm laser with anti-GFP MAb tagged with Alexa Fluor 647-conjugated anti-mouse IgG secondary antibody, cells expressing E-cadherin-GFP were positive in both the GFP and APC channels (660/20 band path). Human breast adenocarcinoma MCF-7 cell line was negative in both channels. Each anti-GFP antibody (purified) was titrated to determine the ideal concentration to generate the optimum signal-to-noise ratio. All eight MAbs (12E6, 12A6, 8H11, 7F11, 4D5, 4C9, 2G6, and 1G9) recognized the majority of A431 cells expressing E-cadherin-GFP (Fig. 3). Anti-GFP-antibody 7F11 was the only weak performer (Fig. 3). Therefore, all the anti-GFP MAbs were used successfully for FACS.

Immunoblotting native and denatured GFP

We first tested the MAbs for detection of native GFP by a dot blot assay. Purified GFP was spotted onto nitrocellulose membranes. Purified maltose-binding protein was spotted as

a negative control. The membranes were then incubated overnight with either the GFP MAbs or anti-MBP MAb and counterstained with anti-mouse antibody. Purified MBP was stained by anti-MBP MAb but not by the anti-GFP MAbs (Fig. 4A). Native GFP was stained to varying degrees by the eight anti-GFP MAbs (Fig. 4B), but not by anti-MBP MAb (Fig. 4A). However, all eight MAbs were unable to bind to the GFP band in Western blots (data not shown), which are denaturing, suggesting that all eight anti-GFP MAbs bind to native GFP epitopes in dot blots, but not to denatured epitopes in Western blots. Controls were performed with a commercial anti-GFP-MAb (data not shown).

Immunoprecipitation

Immunoprecipitation was performed against the *Candida albicans* white phase-specific protein Wh11⁽¹²⁾ tagged with GFP in the *a/a* strain P37005-*TETp*-*WH11*-GFP in which *WH11*-GFP is under the regulation of the tetracycline (doxycycline)-inducible promoter *TETp*.⁽¹¹⁾ A control lysate was prepared using *Candida albicans* wild-type strain P37005, which expressed untagged Wh11. Wh11 protein is cytoplasmic and produced at high levels in white *a/a* cells of this strain when

FIG. 4. Dot blot analysis of MAbs against native GFP. Native GFP and the control protein MBP were spotted onto nitrocellular filter paper. (A) MBP protein failed to stain with the anti-GFP MAb 12E6 (upper) but was recognized by anti-MBP P2F1 MAb (lower). (B) GFP protein stained with each of the eight anti-GFP MAbs.

FIG. 5. Immunoprecipitation of GFP-tagged protein with MAbs. Seven of the eight anti-GFP MAbs were tested for IP of GFP-tagged Wh11, a cytoplasmic protein expressed in *MTL*-homozygous white *C. albicans* cells of strain P37005-*WH11-GFP*. Immunoprecipitates were separated on Western blots and visualized. (A) The first lane was loaded with cell lysate alone to provide the position of Wh11-GFP (34 kDa). The next four lanes were immunoprecipitates from lysate by MAbs 12E6, 8H11, 12A6, and 7F11. The lane labeled Mix contained an immunoprecipitate obtained from lysate by a mixture of MAbs 12E6, 8H11, 12A6, and 7F11. The control lane contains untreated lysate. (B) The first lane was loaded with lysate alone. The next three lanes were immunoprecipitates from lysate using MAbs 1G9, 2G9, and 4C9. The lanes labeled Mix and Control were the same as those in A. The lane labeled Mix + Con. Lysate included a lysate contained untagged Wh11 that was treated with the Mix. The very faint bands observed in the controls represented a low level of nonspecific precipitation that occurred in the absence of antibody.

treated with doxycycline.⁽¹³⁾ Immunoprecipitation was performed with cell lysates of MAbs mixed with Dynabeads linked to Protein G. Seven of the eight anti-GFP MAbs were tested. The immunoprecipitates were analyzed by Western blots for Wh11-GFP (Fig. 5). Lane 1 was loaded with cell lysate containing Wh11-GFP to identify the location of Wh11-GFP. The MAbs we generated that were effective in IP were 8H11, 12A6, 1G9, 2G6, and 4C9 (Fig. 5). A mixture of 12E6, 8H11, 12A6, and 7F11 (Mix) was most effective (Fig. 5). The individual antibodies with the highest performance were 12A6 and 4C9.

Chromatin immunoprecipitation

Finally, we tested seven of the eight anti-GFP MAbs for chromatin immunoprecipitation using a *C. albicans* strain that

expresses the transcription factor gene *BCR1* with *GFP* under the regulation of the tetracycline (doxycycline)-regulated promoter *TETp*.⁽¹³⁾ Associations of Bcr1-GFP to the target promoters of the genes *TCC1* and *PGA31*⁽¹⁷⁾ were assessed by ChIP with each of the seven purified MAbs. The polymerase chain reaction was performed using the immunoprecipitated DNA as template. The primer pairs employed are listed in Supplementary Table 1. The seven tested MAbs, 12E6, 8H11, 12A6, 1G9, 7F11, 2Gb, and 4C9, were effective in ChIP targeting the promoter of *TCC1*, but with different efficacies (Fig. 6). Similar results were obtained with seven purified MAbs, targeting the promoter of the gene *PGA31* (data not shown). The differences observed in the efficacy of each MAb suggest that accessibility of GFP regions in different chromatin-bound transcription factor complexes differ.

FIG. 6. ChIP analysis of *C. albicans* GFP-tagged transcription factor Bcr1 binding to the promoter region of the gene *TCC1*. PCR was performed with the primer pair TCC1-2F/2R (Supplementary Table 1) and a promoter region of the gene *TCC1* immunoprecipitated by ChIP using the indicated MAbs. The lane labeled Mix contained a mixture of MAbs 12E6, 8H11, 12A6, and 7F11. The control lane included a preparation from ChIP with lysate containing untagged wild-type Bcr1 and Mix. The very faint band in the control lane represented a low level of nonspecific precipitation that occurred in the absence of MAb. *C. albicans* genomic DNA (gDNA) was used as a positive control for PCR. ChIP was performed with MAbs in supernatant using Dynabeads.

TABLE 1. EFFICACY OF EIGHT MABs FOR SIX TESTED CHARACTERISTICS^a

Characteristic	12E6	12A6	8H11	7F11	4D5	4C9	2G6	1G9
Immunostaining paraformaldehyde-fixed GFP-expressing cells	++++	++++	++++	+++	+++	+++	+++	++
Immunostaining sections from paraffin-embedded preparations	++++	++++	++++	++++	—	—	+++	++++
Fluorescent-activated cell sorting	++++	++++	++++	++	++	++++	++++	++++
Dot blot	++++	+++	++++	++++	++++	++++	++++	++++
Immunoprecipitation	—	++++	+++	—	ND ^b	++++	++	+++
ChIP (Sepharose beads/purified MAb)	+++	++++	+++	+++	+	— ^c	— ^c	— ^c
ChIP (Dynabeads/MAB supernatant)	+++	++	++	++++	ND	+++	+++	++

^aData are presented quantitatively. Four pluses (++++) indicate the strongest or highest performance (efficiency), while one plus (+), the weakest performance. A negative (—) indicates no performance.

^bND, not determined.

^cData not shown in Figure 6.

Discussion

The need for a variety of anti-GFP antibodies against both native domains and specific epitopes has expanded with the increased use of GFP-tagged proteins. This is true for a variety of other popular protein tags, such as mCherry. For this reason the Developmental Studies Hybridoma Bank, created as a National Resource by the National Institutes of Health, has undertaken a program for generating batteries of monoclonal antibodies against the most widely used protein tags in cell and molecular biology. Here we report our progress in generating the first battery of antibodies against native GFP. The characterization of these antibodies is accompanied by detailed protocols for immunization, fusion, screening, staining, FACS, dot blot analysis, IP, and ChIP. Providing such detail for the characterization of MABs has become a major concern for users of antibodies distributed by private companies and National Resources. Published characterization of MABs is essential for validating the results of users and assuring reproducibility for other scientists.^(23–25)

Alternative to complete Freund's adjuvant

In immunizing mice against GFP, we successfully employed an alternative to CFA, given the toxicity of the CFA emulsifier, to research animals and investigators alike.^(26,27) This toxicity has led institutional animal care committees to approve its use only with reservation.⁽²⁸⁾ The adjuvant we used combined poly(I:C), an analogue of viral double-stranded RNA (dsRNA), and an activating antibody to CD40. Poly(I:C) activates various cell types through two major dsRNA sensors, melanoma differentiation-associated protein-5 (MDA5), and Toll-like receptor 3 (TLR3).⁽²⁹⁾ Through these receptors, poly(I:C) induces the release of Type 1 interferons, proinflammatory cytokines, and co-stimulatory molecules.^(29,30) CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily,⁽³¹⁾ which activates B cell proliferation, differentiation, immunoglobulin production, cytokine production, and expression of co-stimulatory molecules.⁽³¹⁾ Vaccination with the mixture of poly(I:C) and anti-CD40 had previously been used to generate robust cellular immune responses.^(7,32,33) Here we show the effectiveness of this alternative adjuvant in generating MABs against native GFP.

Characterization of eight anti-GFP monoclonal antibodies

We generated a battery of eight anti-GFP MABs against native GFP that do not bind to denatured GFP on Western blots. All eight MABs identified native GFP in dot blots and stained E-cadherin-GFP fusion protein in fixed preparations of A431 human epidermoid carcinoma cells, as evidenced by overlapping anti-GFP-fluorescence images. Six of the eight MABs identified GFP-expressing neurons in sections from fixed, paraffin-embedded transgenic zebrafish embryos. Seven of the eight MABs were quite effective in recognizing GFP-expressing cells using FACS. Five of seven MABs tested proved effective in immunoprecipitating GFP-tagged cytoplasmic protein, and seven MABs proved effective in ChIP analysis, targeting a DNA-binding, GFP-tagged transcription factor. In the case of ChIP analysis, the anti-GFP antibodies were not uniformly effective, suggesting that the same target in possibly different transcription factor complexes may not be equally accessible or abundant or that a slight conformational change of native GFP occurred due to fixation during transcription factor-DNA cross-linking. In Table 1, the eight MABs are ranked for tested characteristics. It would appear that MABs 12A6 and 8H11 were the best all-around performers.

Concluding Remarks

The newly developed water-soluble adjuvant cocktail proved effective in generating a collection of eight anti-GFP MABs, which included a majority that were effective in all tested characteristics. In some cases, these MABs may change their order of effectiveness based on the conformation of the target they identify. They have been shown to recognize wild-type GFP and enhanced GFP but have not been tested with YFP, BFP, or CFP. However, with continued usage, such information will be added by the DSHB to the hybridoma data sheets and catalog. In addition, to this collection of MABs against native GFP, we are now actively developing a second collection against denatured GFP, which will be effective in Western blot analysis.

Acknowledgments

This research was funded by the Developmental Studies Hybridoma Bank (DSHB), a non-profit National Resource

created by the NIH. All antibodies and their hybridomas can be obtained at cost from the DSHB for use in basic research (<http://dshb.biology.uiowa.edu>).

Author Disclosure Statement

The authors have no financial interests to disclose. The DSHB is a non-profit resource directed by David R. Soll.

References

- Shimomura O, Johnson FH, and Saiga Y: Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusa, *Aequorea*. *J Cell Compar Physiol* 1962;59:223–239.
- Tsien RY: The green fluorescent protein. *Annu Rev Biochem* 1998;67:509–544.
- Cormack BP, Valdivia RH, and Falkow S: FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* 1996;173:33–38.
- Mitra RD, Silva CM, and Youvan DC: Fluorescence resonance energy transfer between blue-emitting and red-shifted excitation derivatives of the green fluorescent protein. *Gene* 1996;173:13–17.
- Dubin M, Fuchs J, Graf R, Schubert I, and Nellen W: Dynamics of a novel centromeric histone variant CenH3 reveals the evolutionary ancestral timing of centromere biogenesis. *Nucleic Acid Res* 2010;38:7526–7537.
- Rothbauer U, Zolghadr K, Muyldermans S, Schepers A, Cardoso MC, and Leonhardt H: A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins. *Mol Cell Proteomics* 2008;7:282–289.
- Sanchez PJ, McWilliams JA, Haluszczak C, Yagita H, and Kedl RM: Combined TLR/CD40 stimulation mediates potent cellular immunity by regulating dendritic cell expression of CD70 in vivo. *J Immunol* 2007;178:1564–1572.
- Llopiz D, Dotor J, Zabaleta A, Lasarte JJ, Prieto J, Borrascua F, and Sarobe P: Combined immunization with adjuvant molecules poly(I:C) and anti-CD40 plus a tumor antigen has potent prophylactic and therapeutic antitumor effects. *Cancer Immunol Immunother* 2008;57:19–29.
- Shulman M, Wilde CD, and Kohler G: A better cell line for making hybridomas secreting specific antibodies. *Nature* 1978;276:269–270.
- Abboud CN, DiPersio JF, Brennan JK, and Lichtman MA: Erythropoietic enhancing activity (EEA) secreted by the human cell line, GCT. *J Supramol Struct* 1980;13:199–209.
- Park HC, Kim CH, Bae YK, Yeo SY, Kim SH, Hong SK, Shin J, Yoo KW, Hibi M, Hirano T, Miki N, Chitnis AB, and Huh TL: Analysis of upstream elements in the HuC promoter leads to the establishment of transgenic zebrafish with fluorescent neurons. *Develop Biol* 2000;227:279–293.
- Srikantha T, and Soll DR: A white-specific gene in the white-opaque switching-system of *Candida albicans*. *Gene* 1993;131:53–60.
- Park YN, and Morschhauser J: Tetracycline-inducible gene expression and gene deletion in *Candida albicans*. *Eukaryotic Cell* 2005;4:1328–1342.
- Yi S, Sahni N, Daniels KJ, Lu KL, Srikantha T, Huang G, Garnaas AM, and Soll DR: Alternative mating type configurations (**a**/alpha versus **a/a** or alpha/alpha) of *Candida albicans* result in alternative biofilms regulated by different pathways. *PLoS Biol* 2011;9:e1001117.
- Borneman AR, Leigh-Bell JA, Yu H, Bertone P, Gerstein M, and Snyder M: Target hub proteins serve as master regulators of development in yeast. *Genes Devel* 2006;20:435–448.
- Srikantha T, Borneman AR, Daniels KJ, Pujol C, Wu W, Seringhaus MR, Gerstein M, Yi S, Snyder M, and Soll DR: TOS9 regulates white-opaque switching in *Candida albicans*. *Eukaryotic Cell* 2006;5:1674–1687.
- Nobile CJ, Fox EP, Nett JE, Sorrells TR, Mitrovich QM, Hernday AD, Tuch BB, Andes DR, and Johnson AD: A recently evolved transcriptional network controls biofilm development in *Candida albicans*. *Cell* 2012;148:126–138.
- Johnson JL, Winterwood N, DeMali KA, and Stipp CS: Tetraspanin CD151 regulates RhoA activation and the dynamic stability of carcinoma cell-cell contacts. *J Cell Sci* 2009;122:2263–2273.
- Kam Y, and Quaranta V: Cadherin-bound beta-catenin feeds into the Wnt pathway upon adherens junctions dissociation: evidence for an intersection between beta-catenin pools. *PLoS One* 2009;4:e4580.
- Slanchev K, Carney TJ, Stemmler MP, Koschorz B, Amsterdam A, Schwarz H, and Hammerschmidt M: The epithelial cell adhesion molecule EpCAM is required for epithelial morphogenesis and integrity during zebrafish epiboly and skin development. *PLoS Genet* 2009;5:e1000563.
- Wang B, Wylie FG, Teasdale RD, and Stow JL: Polarized trafficking of E-cadherin is regulated by Rac1 and Cdc42 in Madin-Darby canine kidney cells. *Am J Cell Physiol* 2005;288:C1411–1419.
- Adams CL, Chen YT, Smith SJ, and Nelson WJ: Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. *J Cell Biol* 1998;142:1105–1119.
- Bjorling E, and Uhlen M: Antibodypedia, a portal for sharing antibody and antigen validation data. *Mol Cell Proteomic* 2008;7:2028–2037.
- Rhodes KJ, and Trimmer JS: Antibodies as valuable neuroscience research tools versus reagents of mass distraction. *J Neurosci* 2006;26:8017–8020.
- Saper CB: An open letter to our readers on the use of antibodies. *J Compar Neurol* 2005;493:477–478.
- Guidelines for the use of adjuvants in research, 2013. <http://oacu.od.nih.gov/ARAC/documents/Adjuvants.pdf>
- Stills HF Jr: Adjuvants and antibody production: dispelling the myths associated with Freund's complete and other adjuvants. *ILAR J* 2005;46:280–293.
- Oscherwitz J, Hankenson FC, Yu F, and Cease KB: Low-dose intraperitoneal Freund's adjuvant: toxicity and immunogenicity in mice using an immunogen targeting amyloid-beta peptide. *Vaccine* 2006;24:3018–3025.
- McCartney S, Vermi W, Gilfillan S, Cella M, Murphy TL, Schreiber RD, Murphy KM, and Colonna M: Distinct and complementary functions of MDA5 and TLR3 in poly(I:C)-mediated activation of mouse NK cells. *J Exper Med* 2009;206:2967–2976.
- Jelinek I, Leonard JN, Price GE, Brown KN, Meyer-Manlapat A, Goldsmith PK, Wang Y, Venzon D, Epstein SL, and Segal DM: TLR3-specific double-stranded RNA oligonucleotide adjuvants induce dendritic cell cross-presentation, CTL responses, and antiviral protection. *J Immunol* 2011;186:2422–2429.
- van Kooten C, and Banchereau J: CD40-CD40 ligand. *J Leukocyte Biol* 2000;67:2–17.

32. Ahonen CL, Doxsee CL, McGurran SM, Riter TR, Wade WF, Barth RJ, Vasilakos JP, Noelle RJ, and Kedl RM: Combined TLR and CD40 triggering induces potent CD8+ T cell expansion with variable dependence on type I IFN. *J Exp Med* 2004;199:775–784.
33. Kurche JS, Burchill MA, Sanchez PJ, Haluszczak C, and Kedl RM: Comparison of OX40 ligand and CD70 in the promotion of CD4+ T cell responses. *J Immunol* 2010;185: 2106–2115.

Address correspondence to:
Prof. David R. Soll
Developmental Studies Hybridoma Bank
The University of Iowa
210 Iowa Ave.
Iowa City, IA 52242-1324
E-mail: david-soll@uiowa.edu

Received: December 6, 2013
Accepted: February 25, 2014