Bestrophin Interacts Physically and Functionally with Protein Phosphatase 2A*

Lihua Y. Marmorstein‡, Precious J. McLaughlin†, J. Brett Stanton‡, Lin Yan‡, John W. Crabb‡§, and Alan D. Marmorstein‡‡¶

From the ‡Department of Ophthalmic Research, Cole Eye Institute, and the §Department of Cell Biology, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

Bestrophin is a 68-kDa basolateral plasma membrane protein expressed in retinal pigment epithelial cells (RPE). It is encoded by the VMD2 gene, which is mutated in Best macular dystrophy, a disease characterized by a depressed light peak in the electrooculogram. Recently it was proposed that bestrophin is a chloride channel responsible for generating the light peak. To investigate its function further, we immunoaffinity purified a bestrophin complex from RPE lysates and identified bestrophin and the β-catalytic subunit of protein phosphatase 2A (PP2A) as members of the complex by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Protein-protein interaction between bestrophin and PP2Ac and the structural subunit of PP2A, PR65, was confirmed by reciprocal immunoprecipitation. The C-terminal cytoplasmic domain of bestrophin was sufficient for the interaction with PP2A as demonstrated by a pulldown assay using a fusion of this domain with glutathione S-transferase. Bestrophin was phosphorylated when expressed in RPE-J cells and this phosphorylation was sensitive to okadaic acid. Purified PP2A effectively dephosphorylated bestrophin in vitro. These data suggest that bestrophin is in the signal transduction pathway that modulates the light peak of the electrooculogram, that it is regulated by phosphorylation, and that phosphorylation of bestrophin is in turn regulated by PP2A.

Best macular dystrophy (BMD), 1 a vitelliform macular dystrophy, is an autosomal dominant inherited disorder. Clinically, BMD is characterized by an “egg yolk” or vitelliform lesion in the macula easily visible by fundus examination (1, 2). It is thought that the vitelliform lesion may be caused by the abnormal deposition of lipofuscin in the retinal pigment epithelium (RPE) (3). Histopathologically, BMD has been shown to manifest as a generalized RPE abnormality associated with lipofuscin accumulation, regions of geographic atrophy, and deposition of abnormal fibrillar material beneath the RPE (3, 4). Occasional breaks in Bruch’s membrane with accompanying neovascularization have also been reported (3, 4). Many of these features are also found in age-related macular degeneration, the leading cause of blindness in the elderly.

Despite the histopathologic and clinical features described above, the defining characteristic of BMD is a light peak to dark trough ratio in the electrooculogram (EOG) less than 1.5, without aberrations in the clinical electoretinogram (1). Otherwise asymptomatic carriers of BMD-associated mutations will exhibit an altered EOG (5, 6). The EOG is a late response of the eye to light and is generated by a depolarization of the basal plasma membrane of the RPE (7). This depolarization is thought to be caused by a Ca2+-sensitive chloride current (7–9). Induction of the light peak requires a “light peak substance” that is secreted by the neurosensory retina (7). Transduction of the signal that induces the light peak thus requires signaling across the RPE cell from a presumed receptor at the apical surface of the cells to activate one or more chloride channels in the basolateral plasma membrane of the RPE cell. The details of this pathway and indeed the identity of the light peak substance are unknown.

The gene mutated in BMD, VMD2, was identified in 1998 (10, 18). To date, 79 different mutations have been identified in BMD patients (summarized at the VMD2 mutation data base, www.uni-wuerzburg.de/humangenetik/vmd2.html). Of these, 75 are missense mutations resulting in substitutions at 56 different amino acids (10–19). Two mutations are single amino acid deletions (15, 16, 18). One splice site and one frameshift mutation have been reported (13, 15). In addition, three novel missense mutations have been reported in cases of adult onset vitelliform macular dystrophy (11, 15). Like genes identified for other inherited macular disorders, VMD2 mutations are rare in age-related macular degeneration patients (11, 15, 16).

VMD2 encodes a 585-amino acid protein with an approximate mass of 68 kDa (10, 18) which has been designated bestrophin. Bestrophin shares homology with the Caenorhabditis elegans RFP gene family, named for the presence of a conserved arginine (R), phenylalanine (F), proline (P), amino acid sequence motif. Our laboratory has produced antibodies that recognize bestrophin and demonstrated that bestrophin is a plasma membrane protein, localized to the basolateral surface of RPE cells (20) consistent with a role for bestrophin in the generation or regulation of the EOG light peak. Recently, Sun et al. (21) have provided evidence that bestrophin and...
other RFP family members represent a new class of chloride channels, indicating a direct role for bestrophin in generating the light peak.

As a step toward understanding the function of bestrophin and how mutations in the protein result in retinal degenerative disease, we have immunoaffinity purified a bestrophin-containing complex from porcine RPE. Here, we demonstrate that bestrophin interacts physically with the serine/threonine-protein phosphatase PP2A. We also demonstrate that bestrophin is phosphorylated and that this phosphorylation is sensitive to PP2A and okadaic acid (an inhibitor of PP2A). These data provide the first evidence for how bestrophin and potentially the light peak of the EOG are regulated.

**TABLE I**

Peptides matching bestrophin associated with the ~68 kDa gel band

| Peptide no. | MH$_a$* observed | MH$_a$* calculated | Error ppm | Species matched | Peptide sequence |
|-------------|------------------|--------------------|-----------|-----------------|-----------------|
| 1           | 659.3518         | 659.355            | -4.85     | Human/          | AWIGGR          |
|             |                  |                    |           | pig             |                 |
| 2           | 1044.5000        | 1044.556           | -53.61    | Pig             | VSGIDEAAK       |
| 3           | 1163.5348        | 1163.541           | -5.33     | PP2A            | DFWYNLAPHR      |
| 4           | 1245.7097        | 1245.724           | -11.48    | Pig             | YANLGNVILIR     |
| 5           | 1571.7273        | 1571.728           | -0.45     | Pig             | DHRFPYWALENR    |
| 6           | 1657.8541        | 1657.906           | -31.30    | Pig             | RSPFPGSTFPHWSLQK|
| 7           | 1702.7416        | 1702.738           | 2.11      | Pig             | DPFPYWALENRDEANS|
| 8           | 1803.8045        | 1803.817           | -6.93     | Human           | WWNQENLPKPDPR   |
| 9           | 1911.9615        | 1911.958           | 1.83      | Pig             | TVFNLADLEAPHELK |
| 10          | 2081.0693        | 2081.168           | -47.43    | Human           | YANLGNVILRVSUAVYK|
| 11          | 2125.1294        | 2125.156           | -12.52    | Pig             | TRDPVLYGSSLDENMILR|
| 12          | 2354.1456        | 2354.086           | 25.32     | Pig             | SFELLPESAESAEFPQLQGHRV|
| 13          | 2432.0621        | 2432.049           | 5.39      | Pig             | DMYWDPEPHHPPYTAASQSR|
| 14          | 2837.2902        | 2837.283           | 2.54      | Human/          | VAEQLNPFGDDEDEDFNIVDR|
| 15          | 3551.9899        | 3551.833           | 46.71     | Pig             | TVFNLADLEAPHELKKEPLNEPPGM1HALI4K|
| 16          | 3788.6262        | 3788.701           | -19.74    | Pig             | EDMFQPPEEEEAAHTGLGFHGLGLOOSSDHOOPPR|

*Singly protonated peptide mass.

*Indicates peptide sequence confirmed by nanoelectrospray MS/MS.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Western Blots**—Mouse mAb (mAb) E6-6 and rabbit polyclonal antibody 125 recognizing human, porcine, and monkey bestrophin are described elsewhere (20). Monoclonal antibody E6-1 recognizing human, porcine, and monkey bestrophin was produced in the same fusion as E6-6 and exhibits properties identical to those of E6-6 (data not shown). Monoclonal antibody 1D6 recognizing the catalytic subunit of PP2A, mAb 2G9 recognizing an epitope common to all known PP2A B subunits, and a rabbit polyclonal IgG recognizing the A subunit of PP2A were obtained from Upstate Biotechnology (Lake Placid, NY). Western blots were performed as described previously (22) using alkaline phosphatase-conjugated secondary antibodies and tetranitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

**Immunoadfinity Isolation of Bestrophin-containing Complexes**—RPE cells were isolated from porcine eyes obtained from Hatfield Quality...
Eyes were bisected posterior to the limbus and the neurosensory retina removed. RPE cells were gently brushed from the eyecup in Ca$^{2+}$/H$^{+}$/Mg$^{2+}$/H$^{+}$-free phosphate-buffered saline using a camel's hair brush. The cells were pelleted by centrifugation at 1,000 g for 5–20 min at 4 °C. RPE pellets were stored frozen at −80 °C.

For immunoaffinity purification, RPE cell pellets from 400 eyes were lysed for 3 h at 4 °C in lysis buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and a 1:100 dilution of protease inhibitor mixture III (Calbiochem). Lysates were centrifuged at 10,000 g for 10 min and the insoluble cell debris discarded. Supernatants were precleared with 2.5 ml of protein A-Sepharose CL-4B/100 ml for 2 h at 4 °C. After washing with low salt wash buffer, the bestrophin-containing complexes were eluted with 100 mM glycine, pH 2.5, and collected into tubes containing 1 M Tris, pH 9.5. Aliquots of samples were removed for direct analysis by Western blot. The remaining sample was concentrated using SDS-quinine sulfate precipitation as described previously (24) and after resuspension in SDS-PAGE sample buffer was resolved by SDS-PAGE on a 10% gel. The gel was stained with Gel Code Blue.
were incubated with protein A beads alone. Bestrophin (Best) IP demonstrates that PR65 is present in immunoprecipitates (nonspecific band in the large scale immunoprecipitations (see Fig. 1). Western blot analysis of porcine RPE lysates (B subunit (PR65) serves to link the catalytic C subunit to a B subunit. Immunoprecipitates were analyzed in comparison with the original lysates. A, immunoprecipitation with anti-PP2Ac mAb 1D6 followed by immunoblotting with anti-bestrophin mAb E6-1. In both human and porcine lysates, anti-PP2Ac antibody 1D6 efficiently coimmunoprecipitated bestrophin and enriched it with respect to the original lysate. B, immunoprecipitation with anti-bestrophin mAb E6-1 followed by immunoblotting with anti-PP2Ac mAb 1D6. Consistent with Fig. 1, E6-1 recognizing bestrophin efficiently coimmunoprecipitated PP2Ac.

Peptide mass mapping by MALDI-TOF mass spectrometry.

| Peptide no. | MH +* observed | MH +* calculated | Error (ppm) | Amino acid position | Peptide sequence |
|-------------|----------------|------------------|-------------|-------------------|-----------------|
| 1           | 829.4238       | 829.4231         | -9.723      | 191–198           | RGGWGSPG        |
| 2           | 911.4529       | 911.4627         | -10.7440    | 287–293           | RRTDPDFL        |
| 3           | 951.4856       | 951.4868         | 17.6456     | 121–128           | KYGNNANWY       |
| 4           | 951.4856       | 951.5124         | -28.1730    | 279–286           | RGLEPWHTTR      |
| 5           | 951.4856       | 951.5124         | -28.1730    | 280–287           | RGLEPWHTRT      |
| 6           | 1340.6525      | 1340.6639        | -8.5121     | 268–278           | KYSFLQDPAPRR    |
| 7           | 1792.7742      | 1792.7852        | -6.1501     | 59–73             | KSPDTNLYFMDVYDRG|
| 8           | 2655.2268      | 2655.2803        | -20.1556    | 199–223           | RGAAGYTPQDISETFNHANGLTVSRA|

*Singly protonated peptide mass.

Based on sequence in SwissProt accession no. P11493.

FIG. 3. Reciprocal immunoprecipitation of bestrophin with an antibody against PP2A catalytic subunit. To confirm that PP2Ac interacts with bestrophin, reciprocal immunoprecipitations (IP) were performed. Human and porcine RPE lysates were immunoprecipitated with either antibody 1D6 recognizing PP2Ac or E6-1 recognizing bestrophin. Immunoprecipitates were analyzed in comparison with the original lysates. A, immunoprecipitation with anti-PP2Ac mAb 1D6 followed by immunoblotting with anti-bestrophin mAb E6-1. In both human and porcine lysates, anti-PP2Ac antibody 1D6 efficiently coimmunoprecipitated bestrophin and enriched it with respect to the original lysate. B, immunoprecipitation with anti-bestrophin mAb E6-1 followed by immunoblotting with anti-PP2Ac mAb 1D6. Consistent with Fig. 1, E6-1 recognizing bestrophin efficiently coimmunoprecipitated PP2Ac.

FIG. 4. Interaction of bestrophin with the PP2A structural subunit PR65. A, heterotrimeric structure of PP2A. A structural A subunit (PR65) serves to link the catalytic C subunit to a B subunit. Western blot analysis of porcine RPE lysates (B) with polyclonal antibodies against PP2Ac, B subunits (PP2AB), PR65, and bestrophin, demonstrates that PR65 co-migrates with bestrophin on SDS-PAGE. The B subunits exhibit a mass of 50–55 kDa, similar to the major nonspecific band in the large scale immunoprecipitations (see Fig. 1). C demonstrates that PR65 is present in immunoprecipitates (IP) of bestrophin (Best) or PP2Ac but is absent from controls in which lysates were incubated with protein A beads alone.

Mass Spectrometry and Protein Identification—Protein bands excised from the Gel Code Blue-stained gel were destained in 50% acetonitrile, dried in a Speed-Vac, and rehydrated in 15 μl of 30 mm N-ethylmorpholine acetate, pH 8.6, containing 0.1 μg of tryosylphenylalanyl chromomethyl ketone-modified trypsin (Promega, Madison, WI) and incubated overnight at 37 °C (25). Tryptic digests were then extracted with 60% acetonitrile containing 0.1% trifluoroacetic acid (once with 60 μl, twice with 30 μl) and analyzed by MALDI-TOF mass spectrometry as described previously using a PE Biosystems Voyager DE Pro instrument (25). Measured protein masses were used to search the SwissProt, TrEMBL, PIR, and NCBI sequence data bases for protein identifications and data base accession numbers. Bestrophin identifications were confirmed further by manual comparison against the predicted tryptic peptide map derived from the porcine bestrophin amino acid sequence presented in Fig. 2. The amino acid sequence was obtained by nanoelectrospray tandem mass spectrometry using the PE Sciepi API 3000 triple quadrupole electrospray instrument fitted with nanospray interface (MDS Proteomics A/S, Odense, Denmark) as described elsewhere (25).

Partial Porcine Bestrophin cDNA Sequence—A partial cDNA for porcine bestrophin was prepared by reverse transcription-PCR using total RNA isolated from porcine RPE cells. The upstream primer 5’-CCAACCCTGGGGCACACTGTGCTATCCTGGCGC-3’ was designed based on the human cDNA sequence encoding the conserved peptide YANLGNVLILR observed (Fig. 1). The downstream primer 5’-GGCCCGGTACCTAGGAGTGTGCTTCATCCC-3’ was designed based on the porcine bestrophin sequence in an expressed sequence tag containing the C terminus of porcine bestrophin (GenBank accession no. AW480265). The PCR product was sequenced directly.

GST Pulldown Assay—The GST-bestrophin fusion protein (GST-Best) contains amino acids 334–568 of human bestrophin. To create GST-Best, the corresponding region of human bestrophin cDNA was

FIG. 5. GST pulldown assay with the C-terminal domain of bestrophin. Lysates of porcine neurosensory retina were incubated with either a purified fusion of GST and the C-terminal domain of bestrophin (GST-Best) or purified GST, and then proteins were isolated with glutathione-Sepharose. A, Gel Code blue stain of purified GST and GST-Best. Note that GST-Best is not very stable, and substantial breakdown products are visible. B, Western blot of purified GST-Best and GST with mAb E6-1 demonstrating that bestrophin immunoreactivity is only associated with the GST-Best fusion. C, Western blot of glutathione-Sepharose pulldowns from incubated lysates demonstrating that PP2Ac is brought down by GST-Best but not by GST. These data imply that PP2Ac interacts with the cytosolic C-terminal domain of bestrophin.
Bestrophin Interacts with PP2A

Fig. 6. Phosphorylation of bestrophin. RPE-J cells transduced with AdBest at a multiplicity of infection of 0 or 60 were labeled with \(^{32}\)P, in the presence (OA +) or absence (OA -) of 100 nM okadaic acid, an inhibitor of PP2A. After immunoprecipitation with mAb E6-1, bestrophin was resolved by SDS-PAGE, and the phosphorylation status was determined by phosphorimage analysis. Bestrophin was observed to be phosphorylated. Note an increase in the intensity of the bestrophin band by ~25% in the presence of okadaic acid.

amplified by PCR from a human placenta cDNA library, subcloned into the pGEX-4T-2 vector, and verified by sequencing. The fusion protein was expressed in BL21 bacterial cells and purified by affinity chromatography with glutathione-Sepharose 4B as described previously (26). GST-Best and GST bound to glutathione-Sepharose beads (40 µl of 50% slurry containing 0.5 µg of GST-Best) were added to 1 ml of porcine retinal lysates (500 µg of total protein) that had been precleared with 20 µl of glutathione-Sepharose. The excess amount of GST (10 µg) was used as control for nonspecific sticking of PP2A. After incubation for 1 h at 4 °C, the beads were pelleted and washed three times in low salt wash buffer. The beads were then suspended in SDS-sample buffer and analyzed by SDS-PAGE on a 10% gel and Western blot with PP2A antibody ID6.

Cell Culture and in Vitro Phosphorylation—RPE-J cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 4% fetal bovine serum, nonessential amino acids, glutamine, and penicillin/streptomycin at 32 °C in an environment of 95% air and 5% CO\(_2\), as described previously (27, 28). For in vitro phosphorylation assays, RPE-J cells were plated at confluence (3 x 10\(^5\) cells/cm\(^2\)) in six-well multiwell plates. After 24 h of growth at 32 °C, cells were switched to 39.5 °C and transduced with the replication-defective adenovirus AdBest, directing expression of human bestrophin at a multiplicity of infection of 60 as described previously (20). 24 h after transduction, the cells were washed with phosphate-free Dulbecco’s modified Eagle's medium containing 20 mM HEPES and labeled with 32Pi for 1 h with 800 µCi/ml \(^{32}\)P, containing 0 or 100 nM okadaic acid. Cells were then washed with phosphate-buffered saline and scraped from the plate in ice-cold lysis buffer (1% Triton X-100, in 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.2% bovine serum albumin) containing 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture III (Calbiochem), 50 µl of washed PANSORBIN (Calbiochem), and 0 or 100 nM okadaic acid. After 1 h the lysates were centrifuged at 10,000 × g. Bestrophin was immunoprecipitated from the supernatants with mAb E6-1. After SDS-PAGE, gels were dried and exposed for 30 min to a storage phosphor screen. Phosphorimage analysis was performed using a Molecular Dynamics Typhoon 8600 PhosphorImager and Imagequant version 5.1 software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Dephosphorylation—Purified PP2A isolated from human red blood cells as a heterodimer of the catalytic C and structural A subunits was obtained from Upstate Biotechnology. The activity of the purified enzyme was defined in units where 1 unit releases 1 nmol phosphate/min from 15 µM [\(^{32}\)P]phosphorylase A at 30 °C. For the preparation of enzyme used, 1 unit was equal to ~5 µg of protein.

RPE-J cells were transduced with AdBest and labeled with \(^{32}\)P, as above. After immunoprecipitation, the beads were washed once with 20 mM MOPS, pH 7.5, 150 mM NaCl, 1 mM MgCl\(_2\), 1 mM EGTA, 0.1 mM MnCl\(_2\), containing 0.1 mg/ml bovine serum albumin (reaction buffer). The beads were then resuspended in 195 µl of reaction buffer containing 1 mM dithiothreitol, 60 mM 2-mercaptoethanol and divided into three 65 µl aliquots. The following reagents were added to each tube in a 10 µl volume of reaction buffer containing 1 mM dithiothreitol, 60 mM 2-mercaptoethanol, bringing the volume of the reaction mixture to 75 µl. To one aliquot, no PP2A or okadaic acid was added. To a second aliquot 1 unit of purified PP2A containing both the catalytic C and structural A subunits (Upstate Biotechnology) was added. To the third aliquot 1 unit of purified PP2A and 100 nM okadaic acid were added. Aliquots were then digested for 30 min at 30 °C and the reaction terminated by the addition of 4X SDS-sample buffer. Samples were then boiled for 5 min and resolved by SDS-PAGE. Gels were dried and exposed to Kodak X-Omat film. Films were scanned and band intensities determined using Imagequant 5.1 software.

Fig. 7. In vitro dephosphorylation of bestrophin by PP2A. RPE-J cells transduced with AdBest at a multiplicity of infection of 0 or 60 were labeled with \(^{32}\)P, and bestrophin immunoprecipitated from cell lysates as indicated under “Experimental Procedures.” Immunoprecipitates were digested for 30 min at 30 °C with 0 or 1 unit of PP2A and 0 or 100 nM okadaic acid (OA). Bestrophin was then resolved by SDS-PAGE, and the gel was dried and exposed to film. As shown in A, addition of PP2A to the sample resulted in a significant reduction in bestrophin phosphorylation. Addition of okadaic acid to the sample together with PP2A substantially inhibited the PP2A-mediated dephosphorylation of bestrophin. Data in B are the mean ± S.D. for three independent experiments.

RESULTS

Immunofinity Isolation of a Bestrophin Protein Complex—To identify proteins that interact with bestrophin we immunofinity purified a bestrophin-containing complex from lysates of isolated porcine RPE cells using mAb E6-1. As shown in Fig. 1A immunofinity isolates are enriched for bestrophin, and the RPE lysate is effectively depleted of bestrophin. Gel Code Blue staining demonstrates a similar pattern of bands in column eluates from experiments in which the column was washed with either 150 mM NaCl (low salt; Fig. 1B) or more stringent 700 mM NaCl (high salt; Fig. 1C). The major band at ~68 kDa present in both high salt and low salt eluates was determined to be bestrophin by peptide mass mapping of tryptic peptides using MALDI-TOF mass spectrometry. Five peptides matched bestrophin based on the human amino acid sequence, and three additional peptides matched based on the sequence encoded by a porcine bestrophin expressed sequence tag. Two of the peptides were confirmed to match regions of the predicted porcine bestrophin amino acid sequence using tandem mass spectrometry (see Table 1).

Porcine Bestrophin cDNA—To confirm further the identity of this band as authentic porcine bestrophin we obtained a partial cDNA encoding porcine bestrophin by reverse transcription-PCR from porcine RPE RNA. Primers based on the peptide sequences obtained by mass spectrometry were paired with...
primers based on the deduced sequence obtained from the porcine bestrophin expressed sequence tag for the PCR. The deduced amino acid sequence obtained from the PCR product is aligned with the deduced human (GenBank accession no. NM_004183) and mouse (assembled from the Celera data base, accession no. mCG1951) bestrophin amino acid sequences in Fig. 2. As with the mouse, the porcine amino acid sequence diverges significantly toward the C-terminal domain of the protein. Of interest, the last 18 amino acids of the human sequence represent the peptide against which mAbs E6-1 and E6-6 were produced and are identical at 17 of 18 positions in the porcine sequence. In contrast, the mouse sequence in this region is poorly conserved explaining the species specificity of antibodies produced against this region of the protein.

When the peptides identified by mass spectrometry were compared against the predicted tryptic peptide map of porcine bestrophin, 14 matches were made with the pig sequence, further confirming the identity of the ~68 kDa band as porcine bestrophin (Table I). Two of the original peptides matching human bestrophin come from regions of the protein which were not covered by our partial porcine cDNA.

Identification of PP2A as a Component of the Bestrophin Protein Complex—22 additional gel bands in the high and low salt eluates were detected by SDS-PAGE of the bestrophin protein complex. The majority of the bands were determined to be bestrophin fragments that probably arose from proteolysis during purification. One band at ~36 kDa present in both high salt and low salt eluates was identified as the β-catalytic subunit of protein phosphatase 2A (PP2A) by matching eight predicted tryptic peptides (Table II). To confirm the interaction between bestrophin and PP2Ac a series of experiments was performed in which either bestrophin or PP2Ac was immunoprecipitated from porcine or human RPE lysates (Fig. 3). When we immunoprecipitated with anti-bestrophin antibody, PP2Ac could be detected by Western blots of the bestrophin immunoprecipitate (Fig. 3, left). When we precipitated with anti-PP2Ac, we could detect bestrophin in the immunoprecipitates (Fig. 3, right). These data confirm that bestrophin and PP2Ac interact.

PP2A is a heterotrimeric enzyme consisting of three subunits. In addition to the catalytic subunit, PP2A includes a structural A subunit (PR65) and one of many possible B subunits that appear to function in compartmentalization of the enzyme (29, 30) (Fig. 4A). PP2A phosphatase activity is entirely in the catalytic subunit and does not require interaction with the other subunits (30). Western blotting of porcine RPE lysates with antibodies recognizing the different PP2A subunits (Fig. 4B) indicates that all three subunits are present in RPE cells. Bestrophin co-migrates exactly with PR65, and the B subunits exhibit masses of 50–55 kDa which co-migrate with a nonspecific band in our large scale immunoprecipitates (see Fig. 1). We did not detect PR65 or a B subunit by MALDI-TOF mass spectrometry perhaps because the signals were not strong enough in the mixture of peptides. We examined the interaction of bestrophin with PR65 by immunoprecipitating bestrophin with mAb E6-1 and blotting with an anti-PR65 polyclonal antibody (Fig. 4C). PR65 was readily detected as a band that co-migrated with authentic PR65 in control samples in which PP2A was immunoprecipitated using a mAb against PP2Ac. We conclude that bestrophin interacts with a typical PP2A complex.

Interaction of the Cytosolic Domain of Bestrophin with PP2A—Bestrophin is predicted to have four membrane-spanning α-helices and a large C-terminal cytoplasmic region. We next sought to determine whether the C-terminal region is responsible for the interaction between bestrophin and PP2Ac using a GST pulldown assay. In this experiment, we incubated a porcine retina lysate with a fusion of GST and the C-terminal domain of bestrophin (GST-Best), or with GST alone (Fig. 5A). Porcine retinal lysates were used instead of porcine RPE lysates because neurosensory retina does not express endogenous bestrophin. Thus, all of the PP2A in retina is free of bestrophin prior to incubation with GST-Best. GST-Best or GST and their associated proteins were isolated from the lysates with glutathione-Sepharose and then blotted with an antibody recognizing bestrophin (Fig. 5B) or PP2Ac (Fig. 5C). PP2Ac immunoreactivity was present only in the sample incubated with GST-Best and not in the sample from lysates incubated with GST alone (Fig. 5C). These data further confirm the interaction of bestrophin and PP2A and imply that this interaction occurs with the cytosolic C-terminal region of bestrophin.

Phosphorylation of Bestrophin—PP2A is a serine/threonine phosphatase. The interaction of bestrophin with PP2A suggests that bestrophin might be phosphorylated and that the interaction with PP2A may play a role in regulating the status of bestrophin phosphorylation. To test this hypothesis we performed an in vitro labeling with 32P, in RPE-J cells expressing human bestrophin. Labeling was performed in the presence or absence of 100 nM okadaic acid, an inhibitor of PP2A. After lysis of the cells, bestrophin was immunoprecipitated and its phosphorylation status determined by phosphorimage analysis (Fig. 6). Bestrophin was found to be readily labeled with 32P, indicating that bestrophin is phosphorylated. A strong signal was present in the sample in which phosphatase inhibitors were omitted, suggesting that bestrophin phosphorylation is relatively stable. The addition of okadaic acid to the media during labeling and to the lysis buffer enhanced the bestrophin phosphorylation signal by 46 ± 17% (mean ± S.D., n = 4), implying that PP2A is responsible for the dephosphorylation of a portion of phosphorylated bestrophin. To test further the ability of PP2A to dephosphorylate bestrophin, purified PP2A was used in an in vitro dephosphorylation assay (Fig. 7). The addition of 1 unit of PP2A to a 32P-labeled bestrophin immunoprecipitate resulted in the reduction in the intensity of the bestrophin band of 94.9 ± 0.4% (n = 3) relative to the control. The addition of 100 nM okadaic acid to the same reaction resulted in the preservation of 57 ± 14% (n = 3) of bestrophin band intensity. These data suggest that bestrophin phosphorylation is regulated by PP2A.

Discussion

The amino acid sequence of bestrophin offers few clues to assist in understanding the function of the protein (12). We took the approach that by immunoaffinity-isolating bestrophin we could identify protein-binding partners that could assist in identifying its physiological role in the RPE cell. To this end we have isolated a bestrophin-containing protein complex from porcine RPE and identified PP2A as a member of the complex. Reciprocal immunoprecipitation experiments have confirmed that the interaction between bestrophin and PP2A is significant and not the result of protein contamination of our samples or background. We have also provided evidence that PP2A interacts with the C-terminal domain of bestrophin, that bestrophin is phosphorylated in vitro, and that PP2A can function to regulate the phosphorylation of bestrophin.

Addition of the PP1/PP2A inhibitor okadaic acid to RPE-J cells during labeling and in the lysis buffer had some effect on the phosphorylation status of bestrophin compared with a control sample in which phosphatase inhibitors were omitted (Fig. 6). Furthermore, the addition of purified PP2A to phospho-bestrophin resulted in the near complete dephosphorylation of
phorylation and the light peak may help to explain how mutations in bestrophin result in BMD.

Acknowledgments—We thank George Hoppe and Nicole Kyle for assistance in preparing porcine RPE and Zhenglin Yang for DNA sequencing.

REFERENCES

1. Cross, H. E., and Bard, L. (1974) Am. J. Ophthalmol. 77, 46–50
2. Gass, D. J. (1975) in Stereoscopic Atlas of Macular Diseases: Diagnosis and Treatment (Gass, D. J., ed.) Vol. 1, pp. 305–313, Mosby, St. Louis
3. Weingeist, T. A., Kobrin, J. L., and Watzke, R. C. (1982) Arch. Ophthalmol. 100, 1108–1114
4. O'Grady, S., Fherty, W. A., Fishman, G. A., and Berson, E. L. (1988) Arch. Ophthalmol. 106, 1261–1268
5. Maloney, W. F., Robertson, D. M., and Duboff, S. M. (1977) Arch. Ophthalmol. 95, 793–798
6. Bard, L. A., and Cross, H. E. (1975) Trans. Am. Acad. Ophthalmol. Otolaryngol. 79, OP85–OP873
7. Gallemore, R. P., Hughes, B. A., and Miller, S. S. (1998) in The Retinal Pigment Epithelium (Marmorstein, A. D., Bonilha, V. L., Chiflet, S., Neill, J. M., and Rodriguez-Boulan, E., eds.) pp. 175–198, Oxford University Press, New York
8. Gallemore, R. P., and Steinberg, R. H. (1993) J. Neurophysiol. 70, 1669–1680
9. Gallemore, R. P., and Steinberg, R. H. (1989) J. Neurosci. 9, 1957–1984
10. Petrukhin, K., Koisti, M. J., Bakall, B., Li, W., Xie, G., Marknell, T., Sanberg, O., Forsman, K., Holmgren, G., Andreason, S., Vujic, M., Borgen, A. A., McGarty-Dugan, V., Figueroa, D., Austin, C. P., Metzker, M. L., Caskey, C. T., and Wadelius, C. (1999) Eur. J. Biochem. 266, 241–247
11. Allikmets, R., Seddon, J. M., Bernstein, P. S., Hutchinson, A., Atkinson, A., Sharma, S., Gerrard, B., Li, W., Metzker, M. L., Wadelius, C., Caskey, C. T., Dean, M., and Petrukhin, K. (1999) Hum. Genet. 104, 449–453
12. Bakall, B., Marknell, T., Ingvast, S., Koisti, M. J., Sandgren, O., Li, W., Borgen, A. A., Andreason, S., Rosenberg, T., Petrukhin, K., and Wadelius, C. (1999) Hum. Genet. 104, 383–389
13. Caldwell, G. M., Kalkut, L. E., Griesinger, I. B., Simpson, S. A., Nowak, N. J., Small, K. W., Maumenee, I. H., Rosenfeld, P. A., Sieving, P. A., Shows, T. B., and Ayyagari, R. (1999) Genomics 58, 98–101
14. Eklundh, L., Bakall, B., Wadelius, C., and Andreason, S. (2001) Ophthalmic Genet. 22, 107–115
15. Kramer, F., White, K., Pauleikhoff, D., Gehrig, A., Passmore, L., Rivera, A., Rudolph, G., Kellner, U., Andressi, M., Lorenz, B., Rohrschneider, K., Blankenagel, A., Jurkies, B., Schilling, H., Schutt, F., Holz, F. G., and Weber, B. H. (2000) Eur. J. Hum. Genet. 8, 286–292
16. Lotery, A. J., Munier, F. L., Fishman, G. A., Weleber, R. G., Sieving, P. A., Shows, T. B., and Ayyagari, R. (1999) Genomics 58, 98–101
17. Marchant, D., Gogat, K., Bouthoul, S., Pequignot, M., Sternberg, C., Dureau, P., Deutinger, O., Utne, Y., Hache, J., Puech, B., Puech, V., Demuynck, V., Mouillon, M., Munier, F. L., Schorderet, D. F., Marsac, C., Dufer, J. L., and Ahitbol, M. (2001) Hum. Mutat. 17, 235
18. Marquardt, A., Stohr, H., Passmore, L. A., Kramer, F., Rivera, A., and Weber, B. H. (1998) Hum. Mol. Genet. 7, 1517–1525
19. Palomba, G., Rozzo, C., Angius, A., Pierrotte, C. O., Ozraslesi, N., and Pirastu, M. (2000) Am. J. Ophthalmol. 129, 260–262
20. Marmorstein, A. D., Marmorstein, L. Y., Rayburn, M., Wang, X., Hollyfield, J. G., and Petrukhin, K. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12758–12763
21. Schaefer, R., Ounshi, T., Yao, K. W., and Nathans, J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 4008–4013
22. Marmorstein, L. Y., Ouchi, T., and Aaronsen, S. A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13869–13874
23. Harlow, E., and Lane, D. (1988) Antibodies: A Laboratory Manual, 1st Ed., pp. 521–527, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
24. McKinley, M. P., Bolton, D. C., and Prusiner, S. B. (1988) Cell 55, 57–62
25. West, K., Yao, L., Miyagi, M., Crabb, J. S., Marmorstein, A. D., Marmorstein, L. Y., and Crabb, J. W. (2001) Exp. Eye Res. 73, 479–491
26. Marmorstein, L. Y., Kinev, A. V., Chan, G. K., Bochar, D. A., Benyi, H., Epstein, J. A., Yen, T. J., and Shiokhatter, R. (2001) Cell 104, 247–257
27. Nahi, I. R., Mathews, A. P., Cohen-Gould, L., Gundersen, D., and Rodriguez-Boulan, E. (1995) J. Cell Sci. 107, 37–49
28. Marmorstein, A. D., Bonilha, V. L., Chiflet, S., Neill, J. M., and Rodriguez-Boulan, E. (1996) J. Cell Sci. 109, 3025–3032
29. Janssens, V., and Goris, J. (2001) Biochem. J. 353, 417–439
30. Vischup, D. M. (2000) Curr. Opin. Cell Biol. 12, 180–185
31. Deay, N., Gammeltoft, S., and Brunak, S. (1999) J. Mol. Biol. 294, 1351–1362
32. Kutuzov, M. A., and Bennett, N. (1996) Eur. J. Biochem. 238, 613–622
33. Gordon, S. E., Brautigan, D. L., and Zimmerman, A. L. (1992) Neuron 9, 739–748

2 L. Y. Marmorstein, P. J. McLaughlin, J. B. Stanton, L. Yan, J. W. Crabb, and A. D. Marmorstein, unpublished observation.
Bestrophin Interacts Physically and Functionally with Protein Phosphatase 2A
Lihua Y. Marmorstein, Precious J. McLaughlin, J. Brett Stanton, Lin Yan, John W. Crabb and Alan D. Marmorstein

J. Biol. Chem. 2002, 277:30591-30597.
doi: 10.1074/jbc.M204269200 originally published online June 10, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204269200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 31 references, 7 of which can be accessed free at
http://www.jbc.org/content/277/34/30591.full.html#ref-list-1