Insertion and Topology of Normal and Mutant Bestrophin-1 in the Endoplasmic Reticulum Membrane

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The vitelliform macular dystrophy type 2 (VMD2) gene mutated in Best macular dystrophy encodes a 585-amino acid putative transmembrane protein termed bestrophin-1. The vast majority of known disease-associated alterations are of the missense type, which cluster near predicted transmembrane domains (TMDs). To investigate bestrophin-1 membrane topology and to assess consequences of point mutations on membrane integration, we have analyzed the insertion of putative TMDs into the endoplasmic reticulum (ER) membrane. Out of six potential TMDs, our data suggest a topological model of bestrophin-1 with four transmembrane-spanning segments and one large cytoplasmatic loop between putative TMD2 and TMD5. Consequently, a relatively hydrophobic segment containing putative TMD3 (aa 130–149) and TMD4 (aa 179–201) is located within the cytoplasm. Furthermore, we show that three out of 18 disease-associated alterations investigated (I73N, Y85H, F281del) reveal measurable effects on membrane insertion suggesting that defective membrane integration of bestrophin-1 may represent a potential disease mechanism for a small subset of Best macular dystrophy-related mutations.

Bestrophin-1 is encoded by the vitelliform macular dystrophy type 2 (VMD2) gene as a 585-amino acid putative integral transmembrane protein localized to the basolateral aspect of the retinal pigment epithelium (RPE) (1). Disease-related mutations in the gene are responsible for the autosomal dominant Best macular dystrophy (BMD) (2, 3) but have also been associated with a minor proportion of cases presenting with adult vitelliform macular dystrophy (4), bull’s eye maculopathy (5), and autosomal dominant vitreoretinocochoroidopathy (6). Thus far, a total of 100 distinct mutations have been identified including 92 in BMD, four in adult vitelliform macular dystrophy, one in bull’s eye maculopathy, and three in autosomal dominant vitreoretinocochoroidopathy patients (for further information see BMD mutation data base). Of these, more than 93% are missense mutations, distributed in a non-random fashion across the highly conserved N-terminal half of the protein.

Clinically, BMD is characterized by striking yellowish lesions in the macular area eventually leading to a decline in central vision at later stages in disease pathology. The disease manifests in teenage years although with reduced penetrance and considerable variability in phenotypic expression. Histopathologically, aberrant remnants of heterogeneous material including proteins, lipids, and lipofuscin-containing particles are found in the central retina at the level of the RPE. Typically, BMD patients display a characteristic electrooculogram (EOG) abnormality with a greatly reduced light peak/dark trough ratio reflecting RPE dysfunction (7).

Accumulating experimental evidence suggests bestrophin-1, but also its highly conserved family members bestrophin-2, bestrophin-3 and bestrophin-4 (8, 9), to be involved in Ca2+-dependent transport of chloride ions across cellular membranes. It is still unclear whether bestrophin-1 acts directly as an EOG light peak-producing chloride channel (Refs. 10–12 and reviewed in Ref. 13) or indirectly as an accessory protein modulating neighboring channel function (14). On this note, studies on Vmd2-deficient mice suggested that the light peak of the EOG may be dependent on voltage-gated calcium channels that in turn may be regulated by bestrophin-1 (15). An alternative function of bestrophin-1 may be its involvement in volume sensitivity of RPE cells (16) raising the possibility that functional aspects of bestrophin-1 other than those thought to be of elementary importance to the generation of EOG responses could be involved in primary disease pathology.

To further clarify the nature of BMD-related pathology, investigation into bestrophin-1 membrane topology is needed. In particular, defining the positions of pathogenic mutations relative to defined topological structures may provide clues as to how these mutations interfere with protein function. While in silico hydropathy analyses of the primary bestrophin-1 amino acid sequence reveal six potential hydrophobic domains, insertion of N-glycosylation and tobacco etch virus protease cleavage sites into the protein suggested that only four of these may properly traverse the membrane, with an additional hydrophobic domain dipping into the lipid bilayer (11). By employing the in vitro translation/translocation Escherichia coli leader peptidase (Lep) gene assay (17, 18), we have sought an alternative experimental approach to study membrane topology of bestrophin-1. This method has been successfully applied for resolving membrane topologies of a great number of proteins.
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(19–21). Lep contains two N-terminal transmembrane domains, H1 and H2, and a catalytic P2 domain at the C terminus. While the H1 domain of Lep has the ability to target and traverse the ER membrane, the transmembrane H2 domain of Lep is replaced in the assay system by putative membrane-spanning peptide fragments and tested for glycosylation at the P2 loop indicating proper membrane insertion of the fragment in question. This approach provides evidence for a model of bestrophin-1 with four TMDs and one large cytoplasmatic loop between putative TMDs 2 and 5. Accordingly, the relatively hydrophobic segments of putative TMD3 and TMD4 are likely located within the cytoplasm. In addition, three out of 18 disease-related mutations located within one of the four putative TMDs (I73N, Y85H, F281del) were found to show significant effects on endoplasmic reticulum membrane insertion. Therefore defective membrane integration of bestrophin-1 may represent a potential disease mechanism for a minor subset of BMD-related mutations.

EXPERIMENTAL PROCEDURES

Bioinformatics Analyses—Similarity searches with bestrophin-1 sequences (GenBank™ accession number NP_004174) were done in public data bases available at the National Center of Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov/) and the University of California, Santa Cruz, CA. Phylogeny tree construction was done with PHYLIP, version 3.5 (22, 23), and TREECON, version 1.3b (24), programs and was based on the Neighbor-Joining method (25).

Topological structure prediction used the TOPPRED II software (26), which is based on the Kyte and Doolittle algorithm (27). The average hydrophobic values of putative TMDs of 20–23 amino acid residues were calculated using the Eisenberg scale (28). An average hydropathy plot of multiple bestrophin-1-related protein sequences was generated by the PEPWIN-DOWALL program with a window of 19 amino acids (27).

DNA Manipulations—Initially, full-length VMD2 was amplified by reverse transcriptase-PCR from human RPE mRNA and directionally cloned into the pCEP4 vector via KpnI and SfiI restriction sites (named pCEP-hVMD2), which were introduced into the reverse transcriptase-PCR product at the respective 5′-ends of forward (5′-CTA GGG TAC CAT GAC CAT CAC TTA CAC AAG-3′) and reverse (5′-TGG CCT TGC CGG CCT GGA ATG TGC TTC ATC CCT GT-3′) primer sequences. Fragments of the VMD2 transcript were PCR amplified from the pCEP-hVMD2 template, digested with BclI/Ndel restriction enzymes, and subsequently inserted into the pGEM1 plasmid carrying a modified Lep gene (29) to replace the H2 domain. For generation of the mutated bestrophin-1 constructs, the disease-related mutations were introduced into pCEP-hVMD2 by site directed mutagenesis (30). The correct sequence of all constructs was verified by direct sequencing.

In Vitro Transcription/Translation in Reticulocyte Lysate—Fusion constructs consisting of the H1 domain of Lep, defined human bestrophin-1 putative TMD fragments, and the Lep P2 loop were PCR-amplified with a Lep-specific forward primer containing the T7 promoter sequence (5′-TAA TAC GAC TCA CTA TAG GGA GAC CAC C-3′) and with a reverse primer (5′-TTA ATG GAT GCC GCC AAT GCG-3′). PCR templates were subsequently transcribed/translated in vitro in the presence of [35S]methionine. The reactions were performed in reticulocyte lysates in the presence or absence of canine pancreatic microsomes (Promega, Mannheim, Germany). The samples were then analyzed by SDS-PAGE in 12.5 or 15% polyacrylamide gels. To confirm glycosylation status, translated products were treated with endoglycosidase H (New England Biolabs, Frankfurt, Germany). Polyacrylamide gels were scanned with a Typhoon 9200 phosphorimaging plate scanner and analyzed by the ImageQuant TL software (Amerham Biosciences, Freiburg, Germany). Glycosylation efficiency was measured by assessing the fraction of the band density of the glycosylated signal as compared with the total band signal (glycosylated and non-glycosylated products from a single reaction).

RESULTS

Phylogenetic Relationship of Bestrophin-1 Sequences—To specifically identify bestrophin-1-related orthologous sequences in vertebrate and invertebrate species and to distinguish those from paralogous family members, all available protein sequences with amino acid identities greater than 25% to human bestrophin-1 were extracted from public data bases (see supplemental Table S1). An unrooted dendrogram of 41 protein sequences from 14 representative invertebrate and vertebrate taxa separates at least four main branches of bestrophin-1-related proteins consequently differentiating between four subfamilies termed bestrophin-1 (VMD2), bestrophin-2 (gene VMD2L1), bestrophin-3 (VMD2L2), and bestrophin-4 (VMD2L3) (Fig. 1). Most of the invertebrate bestrophin sequences could not be attributed to one of the four groups hampering their assignment as orthologous or paralogous proteins relative to bestrophin-1. Overall, the data show that the human bestrophins are more closely related to their phylogenetically conserved orthologues than their human paralogous family members.

Putative Transmembrane Domains of Bestrophin-1 and Its Orthologues—In silico hydrophobicity analysis of human bestrophin-1 suggests six potential transmembrane domains (TMD1 to TMD6) in the respective N-terminal regions with average values ranging from 0.69 to 0.82 (Fig. 2, A and B). The C-terminal half of the protein is predominantly hydrophilic. Similarly, hydropathy plotting of the nine bestrophin-1-related vertebrate orthologues including sequences derived from human, chimpanzee, crab-eating macaques, bovine, dog, mouse, rat, chicken, and zebrafish (Fig. 1) result in a consistent pattern of four major and two minor hydrophobic peaks further supporting six potential membrane-spanning regions for the bestrophin-1 subfamily (Fig. 2C).

Analysis of the Membrane-spanning Potential of Putative TMDs—The membrane-spanning potential of the six putative transmembrane segments of bestrophin-1 was systematically analyzed by the in vitro translation/translocation Lep system (17, 18). Generally, membrane integration is directed by a series of alternating signal anchor (SA) and stop transfer (ST) sequences (31–33). With the H1 domain of the E. coli Lep protein providing an ST and the H2 domain an SA signal, the catalytic P2 loop C-terminal to the H2 domain will
be inserted into the ER lumen where it is accessible to glycosylation (Fig. 3A). Initial control experiments demonstrate a glycosylation efficiency of wild type Lep in the range of 60 ± 4% (Fig. 3B, left). These and all subsequent experiments were done at least three times independently and in each case mean values and appropriate standard deviations were calculated. Overall, the range of glycosylation efficiencies is in good agreement with earlier reports (17, 18, 34). Glycosylated versus non-glycosylated products migrate at an increased molecular weight of ~3 kDa. The specificity of the system was tested by replacing the H2 domain with a hydrophilic C-terminal fragment of bestrophin-1 (amino acids 367–389) unlikely to insert into the membrane. As expected, this construct resulted in a non-glycosylated product (Fig. 3B, middle). Conversely, H2 substitution with a hydrophobic sequence of bestrophin-1 (amino acids 28–50) potentially representing a membrane-spanning sequence, translocates the P2 catalytic site to the ER lumen leading to glycosylation.

FIGURE 1. Phylogenetic analysis of proteins homologous to bestrophin-1. The unrooted dendrogram displays the evolutionary relationship between 41 bestrophin proteins based on an alignment of 317 N-terminal amino acid residues. The support for each phylogenetic group was tested using 1000 bootstrap pseudoreplicates. The numbers at the nodes represents bootstrap values. Accession numbers for the 41 sequences used in the phylogenetic analyses are listed in supplemental Table S1.
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which subsequently may or may not be abolished after endoglycosidase H treatment (Fig. 3B, right).

To individually analyze the SA potential of the six predicted TMDs of bestrophin-1, the Lep H2 domain was consecutively replaced by the respective TMD sequences (Table 1). TMD1, TMD2, TMD5, and TMD6 revealed similar glycosylation efficiencies with mean values between 52 ± 4% and 61 ± 6% (Fig. 3C). With a mean glycosylation of 41 ± 3%, TMD4 was not as efficiently glycosylated as may have been expected from its average hydrophobicity index. Segment TMD3 showed even less glycosylation below 20% efficiency, which is considered background noise in this system (20).

SA and ST Function of Adjacent Bestrophin-1 Fragments Containing Consecutive TMDs—In a second set of experiments, a series of constructs were generated each containing pairs of putative TMDs, namely construct VMD2(28–90aa) including TMD1 and TMD2, VMD2(55–149aa) with TMD2 and TMD3, VMD2(130–201aa) with TMD3 and TMD4, VMD2(176–253aa) with TMD4 and TMD5, and finally VMD2(207–291aa) with TMD5 and TMD6 (Table 1). The two constructs VMD2(28–90aa) and VMD2(207–291aa) yielded non-glycosylated products (average efficiency 16 ± 2 and 0, respectively) suggesting that in each fragment both potential TMDs are correctly inserted into the membrane and consequently direct the catalytic P2 domain toward the cytoplasm (Fig. 3D). In contrast, strong glycosylation was found for fusion fragments VMD2(55–149aa) (61 ± 3%) and VMD2(176–253aa) (50 ± 3%) indicating that in each construct only one of the two putative TMDs is integrated into the ER membrane. Considering the glycosylation efficiencies of individual TMDs (Fig. 3C), those may be suspected to likely represent the TMD2 and TMD5 segments, respectively (Fig. 3D). Fusion fragment VMD2(130–201aa) containing TMD3 and TMD4 showed moderate to low glycosylation (29 ± 7%) barely above the given threshold. This may be due to inefficient SA activity of either TMD3 or TMD4. The glycosylation values of individual TMDs (Fig. 3C) seem to favor TMD4 to traverse the membrane although with reduced efficiency of the SA sequence in the context of the VMD2(130–201aa) construct.

As the sequence context is crucial for the nascent polypeptide to acquire proper topology (35, 36), we generated additional constructs containing three consecutive TMDs (Fig. 3E and Table 1). Glycosylation of fusion constructs VMD2(28–149aa) and VMD2(176–291aa) was below or only slightly above background suggesting an even number of SA/ST sequences to insert into the membrane (Fig. 3E). Similar to the above considerations, TMD3 and TMD4 are likely candidates, which may not or only inefficiently traverse the ER membrane. The remaining constructs VMD2(55–201aa) and VMD2(91–253aa) yielded glycosylated products implying an odd number of SA/ST sequences. Again, this finding is consistent with TMD3 and TMD4 segments failing to efficiently participating in ER membrane insertion (Fig. 3E). In both cases alternative explanations are conceivable if either TMD3 or TMD4 act as true transmembrane domains. This interpretation, however, would contradict the glycosylation results obtained with constructs VMD2(28–149aa) or VMD2(176–291aa), respectively.

Finally, a series of C-terminally truncated constructs was generated replacing the Lep H2 domain with N-terminal contiguous bestrophin-1 sequences containing increasing numbers of putative TMDs (Fig. 3F and Table 1). Fusion constructs VMD2(1–55aa), VMD2(1–90aa), and VMD2(1–149aa) containing in ascending order the potential membrane segments TMD1, TMD1 + TMD2, and TMD1 + TMD2 + TMD3, respectively, reveal glycosylation patterns (Fig. 3F, upper and middle panels) in full agreement with the previous experiments, which included the respective transmembrane domains by itself lacking their neighboring sequence context (Fig. 3, C–E). Extending the bestrophin-1 construct to include hydrophobic segment TMD4 (VMD2(1–201aa)) yields a non-glycosylated product. Again, this is in agreement with the notion that TMD3 and TMD4 may fail to insert into the membrane and thus should remain at the cytoplasmic side of the membrane.
The glycosylation pattern of construct VMD2(1–253aa), additionally containing TMD5, and construct VMD2(1–291aa) comprising the entire N-terminal half of bestrophin-1, is also best explained by assuming that TMD3 and TMD4 do not or only inefficiently traverse the ER membrane, while TMD1, TMD2, TMD5, and TMD6 represent functional SA and ST sequences in vitro and therefore may act as authentic transmembrane domains in vivo (Fig. 3F).

Effects of BMD-related Mutations on Bestrophin-1 Topology—Approximately one-third of known BMD-associated mutations cluster within or in close proximity to predicted bestrophin-1 TMDs. To examine possible effects of these point mutations on membrane topology, we introduced a total of 18 disease mutations in various constructs and tested those in the in vitro translation/translocation Lep system (Fig. 4A and Table 1).

By in vitro mutagenesis, point mutations Y29H, L41P, I73N, Y85H, L140R, A146K, A195V, I201T, S231R, T237R, F276L, and F281del were introduced into the respective individual TMD sequence constructs. Compared with normal TMD sequences (Fig. 3C), missense mutations I73N and Y85H in TMD2, L140R in TMD3, and deletion F281del in TMD6 significantly alter the glycosylation pattern by individually abolishing insertion of the mutated fragments into the ER membrane (Fig. 4B). Mutations W24C, R25Q, and S27R immediately flanking TMD1 at the N-terminal side as well as mutations Q293K, N296H, and E300K immediately flanking TMD6 at the C-terminal side do not exert measurable effects on ER membrane insertion (Fig. 4C).

Mutations exerting effects in individual TMD analyses (I73N, Y85H, L140R, and F281del) were re-investigated in extended sequence constructs containing neighboring sequences and adjacent TMDs (Table 1). To this end, the effects of the I73N mutation embedded in a native peptide environment containing TMDs 1, 2, and 3 could not be replicated (Table 1). In contrast, the L140R mutation displayed significant effects on membrane-spanning potential in constructs including TMDs 2 and 3 or TMDs 2, 3, and 4 but not in a sequence context containing TMDs 3 and 4. The Y85H mutation embedded in its native sequence context extending from amino acids 55–149 revealed significantly less glycosylation efficiency (37 ± 2) than its wild type counterpart (61 ± 3) (Fig. 4D). In a construct with the Y85H mutation in the context of the first three consecutive TMDs (VMD2(28–149aa)) (Table 1), no significant glycosylation differences to the non-mutated control were found. Mutation F281del exerted an effect on membrane integration, increasing the glycosylation efficiency from 0 in wild type to 11 ± 2 in the mutant construct containing TMDs 5 and 6 (VMD2(207–291aa)). Likewise, in a construct containing TMDs 4, 5, and 6 (VMD2(176–291aa)) the glycosylation efficiency was reduced from 25 ± 2 in wild type to 17 ± 2 in the mutant counterpart. Concluding, we provide experimental evidence that the Y85H mutation in TMD2 and the F281del mutation in TMD6 may significantly affect membrane integration. Similarly, L140R in TMD3 also displays measurable effects on membrane insertion properties, although its pathological effect remains unclear as TMD3 is not likely to represent a membrane-spanning domain.

DISCUSSION

Our data suggest a topological model of bestrophin-1 with four out of six potential protein segments traversing the membrane including putative TMDs 1, 2, 5, and 6. This leaves a large cytoplasmatic loop of 139 amino acids between TMD2 and TMD5 containing hydrophobic domains TMD3 and 4. Accordingly, a short N terminus of 27 amino acids and a long C-terminal region of 294 amino acid residues both would be localized to the cytoplasmatic side of the membrane (Fig. 5A). This model explains best the results obtained by substituting the H2 domain of the translation/translocation E. coli Lep system with a series of bestrophin-1 fragments containing single or multiple potential TMDs in its natural sequence environment. It is also in agreement with in silico hydrophobicity and hydropathy data, which reveal high phylogenetic conservation throughout the vertebrate orthologues of bestrophin-1.

An alternative transmembrane topography of bestrophin-1 was proposed by Tsunenari et al. (11) based on experimental evidence obtained from insertion mutagenesis of N-linked glycosylation sites and tobacco etch virus protease cleavage sites. Similar to our model, the authors suggest cytosolic N and C termini as well as four segments to traverse the membrane. Conflicting interpretations concern the putative transmembrane domains TMD4 and 5 (Fig. 5B). While our data best fit a model where TMD4 is part of a hydrophobic intracellular loop, TMD5 should be integral to the membrane. In contrast, the model by Tsunenari et al. (11) assumes a more complex topology with TMD4 traversing the membrane followed by TMD5, which is suggested to reside on the extracellular side by dipping into the lipid bilayer. As a consequence, these differences in the two suggested models would lead to differences in the number of extracellular loops. Accordingly, the model presented here predicts two extracellular loops (loop1ext from 51 to 67 aa and loop2ext from 254 to 268 aa), while Tsunenari et al. (11) suggest three such loops (loop1ext from 56 to 73 aa, loop2ext from 200 to 234 aa, and loop3ext from 261 to 270 aa). Alternatively, Tsunenari et al. (11) point out that TMD5 could also reside entirely beyond the membrane participating in an extracellular loop that would comprise amino acid residues 200–270. This is contrasting our results, which demonstrate that TMD5 has a strong membrane spanning potential with a glycosylation value of 58 ± 6% which is significantly higher than that of TMD4 (41 ± 3%). In addition, assuming membrane-traversing features for TMD4 but not TMD5 would clearly conflict with some of our glycosylation data, in particular with those obtained with constructs TMD5, VMD2(207–291aa), VMD2(55–201aa), and VMD2(1–201aa). At present, the dissenting results obtained by the two experimental approaches cannot be resolved. It should be noted, however, that both techniques employ artificial in vitro systems that are not reflecting physiological conditions as are normally present in vertebrate cells. In particular, the Lep system may not allow the distinction between TMD5 acting as an authentic TMD or as a reentrant loop due to its high hydrophobicity. Similarly, mutational insertion of glycosylation sites or tobacco etch virus protease cleavage sites may alter proper...
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TABLE 1
Constructs of normal and mutated bestrophin-1 TMDs

| Construct | TMD | Bestrophin-1 residues | Glycosylation |
|-----------|-----|-----------------------|---------------|
| VMD2-hydrophobic | 0   | 367–389               | 0             |
| VMD2-hydrophobic | 4   | 179–201               | 41 ± 3        |
| TMD1       | 1   | 28–50                 | 61 ± 6        |
| TMD2       | 2   | 68–90                 | 52 ± 4        |
| TMD3       | 3   | 130–149               | 18 ± 3        |
| TMD4       | 4   | 179–201               | 41 ± 3        |
| TMD5       | 5   | 231–253               | 58 ± 6        |
| TMD6       | 6   | 269–291               | 57 ± 4        |
| VMD2(28–90aa) | 1   | 28–90                 | 16 ± 2        |
| VMD2(55–149aa) | 2   | 55–149               | 61 ± 2        |
| VMD2(130–201aa) | 3   | 130–201             | 29 ± 7        |
| VMD2(176–253aa) | 4   | 176–253             | 50 ± 3        |
| VMD2(207–291aa) | 5   | 207–291             | 0            |
| VMD2(28–149aa) | 1–3 | 28–149             | 19 ± 6        |
| VMD2(55–201aa) | 2–4 | 55–201             | 46 ± 3        |
| VMD2(91–253aa) | 3–5 | 91–253             | 45 ± 2        |
| VMD2(176–291aa) | 4–6 | 176–291            | 25 ± 2        |
| VMD2(1–55aa) | 1   | 1–55                 | 60 ± 5        |
| VMD2(190aa) | 1 + 2 | 1–90             | 27 ± 5        |
| VMD2(1–149aa) | 1–3 | 1–149             | 23 ± 6        |
| VMD2(1–201aa) | 1–4 | 1–201             | 16 ± 3        |
| VMD2(253–550aa) | 1–5 | 253–550          | 53 ± 4        |
| VMD2(269–300aa) | 6   | 269–300            | 57 ± 4        |

| Construct + mutation | TMD | Bestrophin-1 residues | Glycosylation |
|---------------------|-----|-----------------------|---------------|
| TMD1-Y29H           | 1   | 28–50                 | 58 ± 6        |
| TMD1-L141P          | 1   | 28–50                 | 56 ± 11       |
| TMD2-I73N           | 2   | 68–90                 | 44 ± 3        |
| TMD2-Y85H           | 2   | 68–90                 | 32 ± 3        |
| TMD3-L140R          | 3   | 130–149               | 1 ± 1         |
| TMD3-L146K          | 3   | 130–149               | 13 ± 5        |
| TMD4-A195V          | 4   | 179–201               | 39 ± 5        |
| TMD4-I201T          | 4   | 179–201               | 36 ± 6        |
| TMD5-S231R          | 5   | 231–253               | 56 ± 5        |
| TMD5-T237R          | 5–6 | 231–253              | 58 ± 5        |
| TMD6-F276L          | 6   | 269–291               | 60 ± 5        |
| TMD6-F281del        | 6   | 269–291               | 45 ± 4        |
| VMD2(1–55aa)-W24C   | 1   | 1–55                 | 59 ± 6        |
| VMD2(1–55aa)-R25Q   | 1   | 1–55                 | 61 ± 3        |
| VMD2(1–55aa)-S27R   | 1   | 1–55                 | 56 ± 8        |
| VMD2(269–300aa)-Q293K | 6   | 269–300             | 51 ± 7        |
| VMD2(269–300aa)-N296H | 6   | 269–300             | 58 ± 2        |
| VMD2(269–300aa)-E300K | 6   | 269–300             | 53 ± 6        |
| VMD2(28–149aa)-Y85H | 1–3 | 28–149             | 29 ± 12       |
| VMD2(55–149aa)-Y85H | 2–3 | 55–149             | 52 ± 4        |
| VMD2(130–201aa)-L140R | 3–4 | 130–201            | 30 ± 3        |
| VMD2(55–149aa)-Y85H | 2–3 | 55–149             | 56 ± 2        |
| VMD2(28–149aa)-Y85H | 1–3 | 28–149             | 37 ± 2        |
| VMD2(207–291aa)-F281del | 5–6 | 207–291         | 11 ± 2        |
| VMD2(176–291aa)-F281del | 4–6 | 176–291        | 17 ± 2        |

* The number indicates which putative TMDs of bestrophin-1 are included in the construct.

by positively charged amino acids. As was shown for comparable mutational changes in the cystic fibrosis transmembrane conductance regulator (39), TMD-associated mutations in bestrophin-1 may also have an impact on folding efficiency. This is in line with previous findings showing that Y85H mutant bestrophin-1 exerts an inhibitory effect on whole cell currents when transiently expressed in HEK 293 cells (10). Furthermore, the Y85H mutation is localized within a core region, which is thought to be a functionally active pore of the putative chloride-conducting bestrophins (13, 40, 41). Anion selectivity analysis by cysteine substitutions of a region spanning murine bestrophin-2 from residue Cys-69 to Asp-104 revealed that in bestrophin-2 the selectivity filter is distributed over ~20 amino acid residues within TMD2 (41). Interestingly, one of the cysteine mutations found to play a potential role in anion permeation is the tyrosine residue at position 85 in murine bestrophin-1. Together these data underline the key role of the tyrosine residue in both the assembly of membrane topology and the conductance of ions across the membrane.

In summary, our data provide support for a working model of bestrophin-1 topology with four of the putative six hydrophobic segments traversing the membrane orienting the N and C termini to the cytoplasm. This model further predicts a relatively hydrophobic intracellular loop between TMDs 2 and 5, which is composed of the putative TMDs 3 and 4. Due to the inability to sufficiently insert into the membrane the latter two protein processing or lead to partially misfolded protein structures locally affecting distinct sequence properties. In this context, it may be of interest to compare the functionality of wild type bestrophin-1 and bestrophin-1 modified after insertional mutagenesis. A functional test of choice could be whole cell measurement of individual transmembrane segments in the presence (+) or absence (−) of ER membranes. Numbers represent glycosylation efficiencies averaged from at least three independent experiments. Sketches below provide an interpretation of the respective experimental results. A, schematic representation of the Lep construct containing the H1 and H2 transmembrane domains that function as ST and SA sequences, respectively, and the glycosylation-sensitive site at the F2 region oriented to the lumens of the ER. B, control experiments demonstrating glycosylation efficiency of the original Lep construct (left) and after replacement of the H2 domain with a hydrophilic (367–389 aa) (center) and a hydrophobic (28–50 aa) (right) bestrophin-1 domain. Endoglycosidase H treatment confirms the upper band as the glycosylated molecule. C, analysis of individual TMDs. D, analysis of adjacent pairs of putative TMDs. E, analysis of three consecutive putative TMDs. F, analysis of C-terminally truncated Lep/bestrophin-1 constructs containing an increasing number of TMDs in their local sequence environment. Results from phorimager quantification of the depicted SDS-PAGE gels are shown in the middle panel. Glycosylated and non-glycosylated products are indicated by filled and open circles, respectively.
hydrophobic segments were excluded as membrane-spanning domains. The experimental system used in this study has the advantage that hydrophobic fragments can be analyzed individually or embedded in their local protein environment thus providing a valuable tool to verify measurable effects in an increasingly native sequence context. In an effort to begin to clarify the molecular pathology of BMD-related mutations, we have further shown that in some cases even subtle changes involving...

FIGURE 4. Analysis of membrane insertion of TMD constructs carrying BMD-related mutations. A, the location of 18 Best disease-related mutations is indicated relative to the six putative TMDs of bestrophin-1. B, mutated and wild type (wt) Lep/bestrophin-1 constructs were analyzed for individual putative TMDs. C, analysis of missense mutations in close neighborhood to TMD1 and TMD6. D, analysis of membrane insertion of mutations Y85H and F281del in an extended context of local sequence environment. Each bar represents the mean value of glycosylation efficiencies obtained in at least three independent experiments. Student’s t test was performed to identify variations between different constructs and values of significance are given as p < 0.05 (*) and p < 0.001 (**).

FIGURE 5. Proposed membrane topology models of bestrophin-1. A, based on the data presented in this study we propose a model in which four of the potential six segments traverse the membrane (TMDs 1, 2, 5, and 6). Putative TMDs 3 and 4 do not traverse the membrane but instead are thought to participate in a relatively hydrophobic loop. B, for comparison, the bestrophin-1 model suggested by Tsunenari et al. (11) is given. The main differences concern TMDs 4 and 5, while the N and C termini are both located on the cytosolic side in the two suggested models.
replacement of a single amino acid residue can exert striking effects on membrane-spanning properties. We therefore conclude that a minor proportion of BMD-related mutations may mediate a direct effect on membrane topology by destabilizing TMD structure.

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