JOURNAL OF BACTERIOLOGY, Aug. 2000, p. 4222–4226 Vol. 182, No. 15
0021-9193/00/$04.00+0

Analysis of the Cellular Localization of Bdr Paralogs in *Borrelia burgdorferi*, a Causative Agent of Lyme Disease: Evidence for Functional Diversity

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Received 3 February 2000/Accepted 15 May 2000

The *bdr* (*Borrelia* direct repeat) gene family of the genus *Borrelia* encodes a polymorphic group of proteins that carry a central repeat motif region containing putative phosphorylation sites and a hydrophobic carboxyl-terminal domain. It has been postulated that the Bdr proteins may anchor to the inner membrane via the C-terminal domain. In this study, we used cellular fractionation methodologies, salt and detergent treatments, and immunoblot analyses to assess the association of the Bdr proteins with the cellular infrastructure in both *Borrelia burgdorferi* (a Lyme disease spirochete) and *B. turicatae* (a relapsing fever spirochete). Triton X-114 extraction and partitioning experiments demonstrated that most Bdr paralogs are associated with the inner membrane-peptidoglycan complex. Analyses of cells treated with the highly chaotropic bile salt detergent deoxycholic acid demonstrated that some Bdr paralogs may also interact with the peptidoglycan, as evidenced by their tight association with the insoluble cellular matrix. In addition, immunoprecipitation (IP) experiments revealed an enhanced IP of all Bdr paralogs when the cell lysates were boiled prior to addition of the precipitating antibody. Furthermore, some Bdr paralogs were accessible to antibody in the IP experiments only in the boiled cell lysates. These observations suggest that different Bdr paralogs may carry out different structural-functional roles. Demonstration of the inner membrane localization of the Bdr proteins and of the differences in nature of the interaction of individual Bdr paralogs with the cell infrastructure is an important step toward defining the functional role of this unique protein family in the genus *Borrelia*.

The borreliae harbor an extraordinary number of plasmid-carried paralogous gene families (1–6, 8, 10, 15). Most of the putative proteins encoded by these paralogous gene families exhibit little or no homology with proteins of known function. This observation, coupled with the absence of a highly developed genetic manipulation system for the borreliae and the difficulties inherent in the genetic manipulation of individual members of large gene families, has complicated efforts to define their functional roles.

The *bdr* genes form a particularly large gene family that encodes a highly polymorphic group of proteins with putative phosphorylation motifs and a membrane-spanning domain. The *bdr* gene family of *Borrelia burgdorferi* B31MI contains 18 members (10). *bdr*-related gene families have been identified in several other *Borrelia* species (2, 3, 5–7, 16, 26, 27), and immunoblot analyses have demonstrated that a variable set of Bdr paralogs are produced by *B. garinii*, *B. burgdorferi*, *B. turdae*, *B. tanukii*, *B. japonica*, *B. valaisiana*, *B. afzelii*, *B. coriaceae*, *B. bissettii*, *B. anserina*, *B. miyamotoi*, *B. parkeri*, *B. hermsii*, and *B. turicatae* (18). The universal distribution and expression of the *bdr* genes is indicative of an important genuswide functional role. Evolutionary analyses of Bdr sequences have demonstrated the existence of six distinct Bdr subfamilies (BdrA through BdrF) in the genus *Borrelia* (5–7). All isolates analyzed to date carry members of at least 2 Bdr subfamilies, suggesting that there may be functional partitioning among Bdr paralogs.

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Bdr proteins possess a stretch of 20 amino acids at their C termini that form a highly hydrophobic region predicted by computer analyses to be membrane spanning (6, 7, 18, 28). The absence of a consensus signal peptide and the presence of a C-terminal hydrophobic domain, which would likely serve as a stop-transfer sequence, suggests that membrane association would most likely be with the inner membrane (IM). To further our understanding of the biological role of the Bdr protein family at the genuswide level, we sought in this study to determine the cellular localization of the Bdr proteins in diverse *Borrelia* species.

MATERIALS AND METHODS

Cultivation of bacterial strains. The clonal populations of infectious *B. burgdorferi* B31MI and *B. turicatae* OZ-1 used in these analyses were generated by subsurface plating of postinfection populations as previously described (6, 7, 24). The Lyme disease and relapsing fever spirochetes were cultivated in BSK-H medium (Sigma) supplemented to 6 and 12%, respectively, with rabbit sera (Sigma). Bacteria were harvested by centrifugation and washed with phosphate-buffered saline (PBS) to remove medium-derived proteins.

Analysis of the nature of the association of the Bdr proteins with *B. burgdorferi* and *B. turicatae*. *Borrelia* cells were salt treated as previously described by Skare et al. (22). In brief, ~1.4×10^9 cells were resuspended in PBS (pH 7.4)–1 M NaCl–0.1 M Na_2CO_3 (pH 11.5) or 1% Triton X-100–1 M NaCl. After incubation for 5 min at room temperature, samples were diluted to 1 ml with PBS, placed on ice for 10 min, and then centrifuged for 1 h at 20,000 × *g* at 4°C. Proteins were precipitated from the supernatant with 100% trichloroacetic acid (TCA; Sigma) as follows. After the addition of 100 μl of TCA, the samples were placed in a −20°C freezer for 15 min and then centrifuged (5 min; 10,000 × *g*). The supernatant was discarded, and the pellet was resuspended in 50 μl of 0.1 N NaOH and 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) solubilizing solution (14) for subsequent SDS-PAGE and immunoblot analyses using anti-Bdr and anti-Fla antisera.

Generation of OMV preparations and subcellular fractions by Triton X-114 extraction and phase partitioning. Cellular fractionation of *B. burgdorferi* B31 by Triton X-114 extraction and phase partitioning were conducted as previously described (9). *B. burgdorferi* B31 outer membrane (OM) vesicles (OMV) were obtained as described by Skare et al. (21).

Treatment of *Borrelia* cells with the bile salt detergent DCA. *B. burgdorferi* B31 was treated with either 2, 3, or 4% deoxycholic acid (DCA; Sigma) as follows. *B. burgdorferi* B31MI cells (2.1×10^9) were washed with PBS, resuspended in 100 μl of DCA, incubated at room temperature for 10 min, and then placed on ice for 5 min. The samples were diluted to 900 μl with PBS and centrifuged (60 min; 18,000 × *g*). The supernatant was removed, and both the pellet and supernatant fractions were saved. The pellet was solubilized in 100 μl of SDS solubilizing (14) solution for subsequent analysis. The proteins present in the supernatant were concentrated by precipitation with 100% TCA as described above.

SDS-PAGE, antisera, and immunoblotting. Proteins were fractionated by electrophoresis in SDS–15% polyacrylamide gels and immunoblotted onto polyvinylidene difluoride (PVDF) membranes by electroblotting as previously described (7). For these analyses, we used anti-Bdr (7), anti-Fla (25), anti-Oms28 (22), anti-p66 (23), and anti-DbpA (13) antisera at dilutions of 1:1,000, 1:1,000 1:2,000, 1:1,700, and 1:5,000, respectively; a dilution of 1:40,000 was used for the secondary antibody. All immunoblots were blocked overnight in blocking buffer (PBS, 0.2% Tween, 0.002% NaCl, 5% nonfat dry milk), incubated with the appropriate antisera at room temperature for 1 h, and washed three times with wash buffer (PBS, 0.2% Tween, 0.002% NaCl). ImmunoPure goat anti-rabbit immunoglobulin G (heavy and light chain) peroxidase-conjugated secondary antibody (Pierce) was incubated with the blots for 1 h at room temperature and then washed three times with wash buffer. For chemiluminescent detection, the Supersignal West Pico Stable Peroxide solution and the Supersignal West Pico Luminol/Enhancer solution were used (Pierce). The immunoblots were exposed to film for 1 to 30 s. The generation and specificity of the anti-Bdr antiserum have been previously described (18). In brief, the antiserum was generated in rabbits using recombinant BdrF. This antiserum reacts with Bdr paralogs from each of the known Bdr protein subfamilies at the genus level.

IP. For the immunoprecipitation (IP) analyses, 3×10^8 cells were washed two times with PBS; after resuspension a third time in PBS, the samples were split in two and pelleted by centrifugation. The bacterial cells were lysed by resuspension in 50 μl of IP buffer (20 mM sodium phosphate [pH 7.5], 500 mM NaCl, 0.1% SDS, 1% NP-40, 1.0% DCA, 0.02% sodium azide). One of the duplicate samples was boiled for 10 min, while the other was held at room temperature. AntiBdr antiserum was added to both samples at a final dilution of 1:1,000, and both were incubated overnight at 4°C. Then 100 μl of UltraLink immobilized protein A/G (Pierce) was added, and the samples were incubated at room temperature for 2 h with gentle rocking. IP buffer was added (0.5 ml), the samples were centrifuged (2,500 × *g*, 3 min), washed seven times with IP buffer (0.5 ml), and washed once with 0.5 ml of water, and then the bound antigen-antibody complexes were pelleted. SDS solubilizing solution (25 μl) was added, the samples were heated (95°C for 5 min) and centrifuged (2,500 × *g* for 3 min), and the supernatant was transferred to a new tube. The pelleted resin was washed one additional time with 25 μl of SDS solubilizing solution and then centrifuged as described above. The supernatants were combined, and 18-μl aliquots were analyzed by SDS-PAGE in a 12% gel.

RESULTS

Analysis of the association of the Bdr proteins with the *B. burgdorferi* cell infrastructure. To assess the interaction of the Bdr proteins with the cellular infrastructure, cells were subjected to a variety of salt treatments that disrupt the OM to various degrees, thereby releasing periplasmic proteins and proteins that are loosely associated with the OM or with the outer leaflet of the IM (22). *B. burgdorferi* and *B. turicatae* cells were treated with either 1 M NaCl, 0.1 M Na_2CO_3, or PBS (negative control), and immunoblot analyses of the precipitated supernatant and pelleted fractions were performed with anti-Bdr, anti-Fla, and anti-DbpA antisera. When treated with 1 M NaCl or 0.1 M Na_2CO_3, all immunoreactivity with the anti-Bdr antiserum occurred with Bdr paralogs present in the pelleted fraction (Fig. 1A). In contrast, treatment of the cells released some Fla (Fig. 1B) and DbpA (data not shown) into the supernatant. The Fla protein, which is a structural component of the endoflagella, is an inner membrane-anchored, periplasmic protein. The release of Fla into the supernatant, but not the Bdr proteins, demonstrates that the Bdr proteins are tightly associated with the cell infrastructure.

Liberation of at least some Bdr protein from the cell required rigorous disruptive measures such as sonication or treatment with Triton X-100 (with 1.0 M NaCl) (Fig. 1A). However, most of the Bdr protein remained with the pellet, indicating that the Bdr proteins are not free cytoplasmic or periplasmic proteins. In contrast, sonication of the cells resulted in the release of a significant percentage of Fla into the supernatant (Fig. 1B). These analyses provide further support for the hypothesized tight association of Bdr proteins with the cellular infrastructure. Of interest is the observation that specific low-molecular-weight (MW) Bdr paralogs are almost completely liberated from the cell upon treatment with Triton X-100–NaCl, whereas other paralogs are not and remain with the pelleted material. This observation indicates that not all Bdr proteins interact with the cell in the same way.

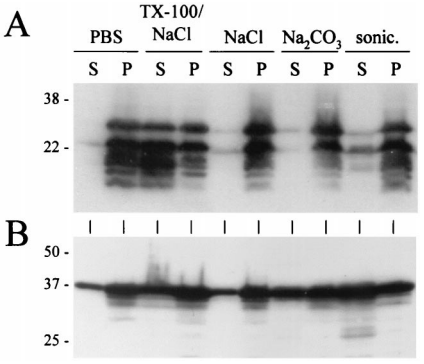

FIG. 1. Treatment of *B. burgdorferi* B31MI cells with salts or detergent to assess interaction of the Bdr proteins with the cell infrastructure. Cell pellets of *B. burgdorferi* B31MI clone 1 were treated with reagents indicated above the lanes (as described in the text). The concentrations of NaCl and Na_2CO_3 used were 1 and 0.1 M, respectively. The abbreviation "sonic." indicates that the cells were disrupted by sonication. After treatment, the samples were centrifuged, and the supernatant (S) and pelleted (P) fractions were collected. Proteins in the supernatant fraction were concentrated by precipitation with TCA. Both the supernatant and pellet fractions were solubilized with SDS-sample buffer, fractionated by SDS-PAGE in a 15% gel, and transferred onto a PVDF membrane for immunoblot analyses as described in the text. The immunoblots were screened with anti-Bdr (A) and anti-Fla (B) antisera. Positions of MW standards are shown to the left in kilodaltons. TX-100, Triton X-100.

Analysis of OMV and use of Triton X-114 extraction and phase partitioning to assess Bdr localization. To assess the possible association of the Bdr proteins with the OM, OMV were isolated and immunoblotted. Bdr proteins were not detected in the OMV (Fig. 2). DbpA (decorin binding protein A) (11), an established OM protein (12), served as a positive control in the immunoblot analyses of the OMV preparations. As expected, the anti-dbpA antiserum reacted strongly with the OMV preparation.

To determine if the Bdr proteins are associated with the IM-peptidoglycan complex (PC), Triton X-114-partitioned cellular fractions were generated and subjected to SDS-PAGE and immunoblotting. Triton X-114 partitioning has been widely used to assess the subcellular localization of spirochetal

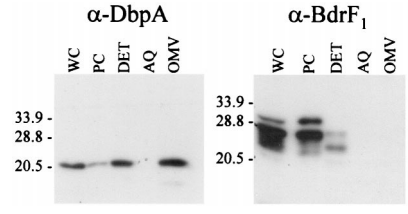

FIG. 2. Immunoblot analyses of OMV preparations and subcellular fractions obtained by Triton X-114 partitioning of *B. burgdorferi* B31MI. The OMV preparations and Triton X-114 fractions were obtained, fractionated by SDS-PAGE, and immunoblotted as described in the text. The antisera used are indicated above the immunoblots. WC, whole-cell lysates; DET, detergent phase; AQ, aqueous phase. Positions of MW standards are indicated to the left in kilodaltons.

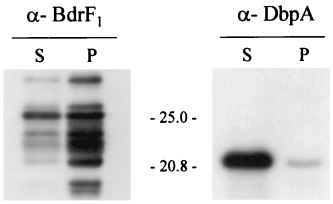

FIG. 3. Immunoblot analysis of *B. burgdorferi* B31MI clone 1 treated with 4% DCA. Cell pellets were resuspended in 4% DCA as described in the text, and supernatant (S) and pellet (P) fractions were obtained. The proteins were fractionated by SDS-PAGE in a 12.5% gel, immunoblotted, and screened with anti-BdrF1 or anti-DbpA antiserum, as indicated. Positions of MW standards are indicated between the panels in kilodaltons.

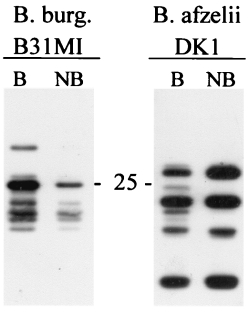

FIG. 4. Immunoblot analysis of Bdr proteins immunoprecipitated from *B. burgdorferi* B31MI clone 1 (left) and *B. afzelli* DK1 (right). IP and subsequent immunoblot analyses were performed as described in the text. The immunoblots show Bdr proteins precipitated from cell lysates that were either held at room temperature (NB [not boiled]) or boiled (B) prior to addition of the anti-Bdr antiserum. The 25-kDa position is marked between the panels.

proteins (9, 17, 19–22). Immunoblot analysis of the Triton X-114 fractions revealed that the majority of Bdr protein was associated with the PC fraction and with the positive control whole-cell lysates. The presence of the Bdr proteins in the PC indicates an IM or cytoplasmic localization. The complete absence of Bdr proteins in the aqueous phase would argue against cytoplasmic localization since at least some degree of leakage of cytoplasmic proteins occurs upon Triton X-114 treatment. To verify the Triton X-114 partitioning results, several controls were performed with antisera targeting proteins of known cellular location and whose behavior upon Triton X-114 partitioning has been demonstrated. Anti-DbpA antiserum (13) reacted with the detergent phase, consistent with the demonstrated lipidation of this protein (12), and anti-Oms28 antiserum reacted specifically with the aqueous phase (data not shown), consistent with earlier reports regarding the fractionation and partitioning of this protein (22). It is important to note that not all Bdr proteins partitioned the same way upon Triton X-114 extraction. While most Bdr paralogs remained with the PC, one partitioned predominantly into the detergent phase. These data support the hypothesis that different Bdr paralogs may carry out different structural-functional roles.

Treatment of *Borrelia* with DCA, a detergent with chaotropic properties: additional evidence for different types of interaction between Bdr paralogs and the cell architecture. To further assess the nature of the interaction of the Bdr proteins with the cellular infrastructure, we treated cells with different concentrations (1, 2, 3, or 4%) of the bile detergent DCA. Due to the chaotropic properties of DCA, treatment with this agent will completely disrupt membranes by disrupting hydrophobic interactions and thereby release membrane proteins into the supernatant. Even after treatment of cells with 4% DCA, a significant proportion of the Bdr protein remained associated with the pelleted material. This indicates that the interaction with the PC is not solely via the insertion of the C-terminal transmembrane domain into the lipid bilayer of the IM. To verify that the membranes were being throughly disrupted upon treatment with 4% DCA, aliquots of the pellet and supernatant fractions were immunobloted and screened with anti-DbpA antiserum to verify the release of the OM protein, DbpA. The majority of the DbpA was found to be present in the supernatant phase, with only minor amounts associated with the pelleted material (Fig. 3), thereby demonstrating the effectiveness of the DCA treatment. It is important to note that at least two of the Bdr paralogs remained exclusively associated with the pelleted material. This important observation indicates that there are different types of interactions between specific Bdr paralogs and the cellular infrastructure. These findings support our early suggestion of functional partitioning among Bdr paralogs (6, 7, 18).

IP analyses of the Bdr proteins. The experiments described above suggested that some Bdr paralogs, in addition to being IM anchored, also interact with other components of the cellular architecture. To test this hypothesis, IP analyses were performed with anti-Bdr antiserum and cell lysates that were either boiled or not boiled prior to addition of the antisera. Subsequent immunoblot analyses of the immunoprecipitated Bdr proteins using the anti-Bdr antiserum revealed that boiling markedly enhanced the IP of all Bdr paralogs (Fig. 4). Furthermore, several Bdr paralogs could be immunoprecipitated only from the boiled samples. The approximate molecular masses of these additional Bdr paralogs in *B. burgdorferi* B31MI were 30, 25.5, and 20 kDa, consistent with the sizes of known Bdr proteins in this cloned isolate. The only Bdr protein in *B. burgdorferi* B31MI with a size near 30 kDa is $BdrF_2$ (30.2 kDa), one of three members of the BdrF subfamily. The other two members of this family are 25.8 kDa ($BdrF_3$) and 19.9 kDa ($BdrF_1$) in size. The other two Bdr paralogs that were immunoprecipitated in the boiled samples are close in size to only $BdrF_3$ and $BdrF_1$. It is important to note, however, that there are other Bdr proteins in B31MI with sizes near 26 and 20 kDa, and so these proteins cannot be definitively identified. To determine if other Lyme disease spirochetes also carry Bdr proteins that exhibit differences in behavior in IP experiments conducted as described above, we analyzed *B. afzelii* DK1; the results obtained were similar: four distinct dominant Bdr paralogs precipitated either with or without boiling. However, four additional minor bands were immunoprecipitated only when the cell lysates were boiled prior to addition of the antisera. Since the composition of the Bdr protein family is largely unknown in isolate DK1, we can reach no conclusions concerning the subfamily affiliation of these paralogs or of their specific identity. While we previously demonstrated that DK1 carries and expresses multiple Bdr alleles (18), only one has been cloned and sequenced (25). The detection of several Bdr paralogs only in the boiled samples indicates that the epitopes recognized by the anti-Bdr antiserum in these specific paralogs are not exposed for antibody binding unless the cell lysates are subjected to harsh disruptive conditions. It can be concluded that these specific Bdr paralogs interact with or are oriented in a fashion in the IM that is different from that of other Bdr paralogs.

DISCUSSION

Determination of the cellular localization of the Bdr proteins is an important step in deciphering their cellular function(s). All Bdr proteins carry a hydrophobic C-terminal domain that is predicted by TMpred analyses to be membrane spanning. The TMpred values for the Bdr proteins are quite significant and are in all cases greater than 2,000 (with 500 considered significant) (6, 7, 18). Based on these features and the absence of an export signal, we previously hypothesized that the Bdr proteins are membrane anchored via their C-terminal domain to the IM (18). To address this hypothesis, we first sought to verify that these proteins are not present in the OM or freely in the periplasm. This was definitively confirmed through immunoblot analysis of OMV preparations and of salt-treated cells. In support of this general finding, an earlier report demonstrated that the Bdr proteins are not accessible to proteinase K in intact *Borrelia* cells (28).

Triton X-114 extraction and partitioning experiments demonstrated that most of the Bdr proteins are associated with the PC. Collectively, the data presented above indicate an association with the PC infrastructure and not the cytoplasm. First is the complete absence of these proteins from the aqueous phase in the Triton X-114-partitioned samples. Second is the almost complete association of the Bdr proteins with the pelleted material (membrane fragments and peptidoglycan) after sonication of the cells. Sonication treatment results in the complete release of all soluble periplasmic and cytoplasmic proteins. Last, most of the Bdr protein remained with the pelleted insoluble material even after treatment of the cells with Triton X-100 or with 4% DCA. Since treatment with DCA completely disrupts the hydrophobic interactions necessary for maintenance of lipid bilayers and hence membrane integrity, it is evident that in addition to the anchoring of the Bdr proteins to the inner membrane, they may also be involved in interactions with other components of the PC, presumably the peptidoglycan.

Not all Bdr proteins behaved in the same way in the phase partitioning and chemical treatment experiments, suggesting that the nature of the interaction of individual Bdr proteins with the cellular infrastructure differs. In the Triton X-114 partitioning experiments, a Bdr protein of ~23 kDa partitioned exclusively into the detergent phase. Since all of the Bdr proteins possess the hydrophobic domain, they should in fact phase partition into the detergent phase. The fact that most do not partition into the detergent phase suggests that most paralogs may interact or complex with other cellular components. Aberrant behavior of specific paralogs was also observed in the IP experiments. Specific paralogs were found to be inaccessible to antibody unless the cells were rigorously disrupted by a combination of detergent treatment and boiling. While this phenomenon was observed in two different *Borrelia* isolates (*B. burgdorferi* B31MI and *B. afzelii* DK1), the molecular weights of the Bdr paralogs that exhibited this property differed in the two isolates analyzed. It is the repeat motif domain that is largely responsible for differences in the MW of Bdr proteins. While this domain is stable over short-term murine infection as well as upon in vitro cultivation (18), it is apparent that this domain is not evolutionarily stable. Some Bdr paralogs that were inaccessible to antibody in the unboiled lysates in the IP analyses also behaved differently in the DCA treatment experiments. Some Bdr proteins exhibited complete or enhanced association with the pelleted insoluble material even after treatment of the cells with 4% DCA. In contrast, other Bdr paralogs partitioned equally into both the pellet and the supernatant. It is important to note that the concentration of DCA (4%) used in these experiments well exceeds that which is necessary for complete dissolution of the membrane and for the total disruption of hydrophobic interactions. This suggests that the interaction between some Bdr paralogs and the insoluble matrix is rather strong and may be covalent in nature.

The number of Bdr paralogs expressed by a cell and the tightness of their MW range make it difficult to differentiate between most of the individual Bdr paralogs detected in the immunoblot analyses. However, some have MWs distinct enough that their identities can be determined. For example, the 30.6-kDa Bdr protein detected in the boiled samples in the IP experiment can only be $BdrF_2$ since no other *B. burgdorferi* B31MI Bdr paralogs have a size in this range. The sizes of other paralogs detected in the IP analyses of boiled samples were estimated to be approximately 26 and 20 kDa, close to the sizes of $BdrF_3$ (25.8 kDa) and $BdrF_1$ (19.9 kDa), respectively. Hence, the paralogs that exhibited unique behavior in the experiments described above appear to belong to a single subfamily. The *bdrF* genes differ from the *bdrD* and *bdrE* genes of *B. burgdorferi* B31MI in that they are all carried on linear plasmids. We have hypothesized that the subdivision of the Bdr proteins into distinct subfamilies may reflect functional segregation among paralogs. Functional diversity may be further enhanced by the multiallelic and polymorphic nature of members of a given subfamily. The analyses described in this report suggest that this hypothesis may be correct and warrants further investigation.

The data presented here suggest that in addition to anchoring to the IM, the Bdr proteins may also interact with insoluble components of the cellular infrastructure. In an earlier study, we hypothesized that the Bdr proteins are largely cytoplasmic but are anchored to the IM via their hydrophobic C-terminal domain (18). While this putative transmembrane domain is variable in sequence, it is important to note that all Bdr paralogs terminate with either a lysine or an asparagine residue. If this residue is exposed in the periplasm, it could be available for interaction with the peptidoglycan, perhaps through a Schiff's base linkage. The tight association of the Bdr proteins with the insoluble cellular matrix supports this possibility. The particularly tight association of $BdrF_3$ and at least two other Bdr paralogs (which also appear to be BdrF subfamily members) with the insoluble matrix may be a consequence of the sequence and physical properties of their C-terminal regions. All BdrF paralogs terminate with the sequence phenylalanine-lysine, while all Bdr E paralogs terminate with isoleucine-serine-lysine.

In summary, we have demonstrated that the Bdr proteins are specifically associated with the PC and are anchored to the IM most likely via their hydrophobic C-terminal domain. In addition, they further interact with the insoluble component of the cellular infrastructure, possibly through their positively charged C-terminal residues. Subtle differences among Bdr paralogs may allow each to fulfill a specific functional or structural role. The demonstration that different paralogs associate with the cell architecture in different ways is important as it provides a possible biological rationale for the necessity to maintain the *bdr* genes as large gene families.

ACKNOWLEDGMENTS

We thank David Blanco, Melissa Caimano, Mark Hanson, Michael Lovett, Medimmune, Jim Miller, Justin Radolf, Scott Samuels, and Ellen Shang for contributing important reagents and for valuable insight.

This work was supported in part by a grant from the Jeffress Trust. We are deeply indebted to the Trust for ongoing support.

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