The *Borrelia burgdorferi* outer-surface protein ErpX binds mammalian laminin

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The Lyme disease spirochaete, *Borrelia burgdorferi*, can invade and persistently infect its hosts' connective tissues. We now demonstrate that *B. burgdorferi* adheres to the extracellular matrix component laminin. The surface-exposed outer-membrane protein ErpX was identified as having affinity for laminin, and is the first laminin-binding protein to be identified in a Lyme disease spirochaete. The adhesive domain of ErpX was shown to be contained within a small, unstructured hydrophilic segment at the protein's centre. The sequence of that domain is distinct from any previously identified bacterial laminin adhesin, suggesting a unique mode of laminin binding.

INTRODUCTION

Borrelia burgdorferi, the Lyme disease spirochaete, is capable of infecting immunocompetent humans and other vertebrates for extensive periods, even for the host's lifetime. *B. burgdorferi* is an extracellular organism, frequently found associated with its hosts' connective tissues (Barthold *et al.*, 1991, 1992a, b, 1993; Cabello *et al.*, 2007; Cadavid *et al.*, 2003; De Koning *et al.*, 1987; Defosse *et al.*, 1992; Franz *et al.*, 2001; Häupl *et al.*, 1993; Kornblatt *et al.*, 1984; Pachner *et al.*, 1995). *B. burgdorferi* has been demonstrated to possess affinity for various host extracellular matrix (ECM) components, such as type I collagen, fibronectin and decorin (Cabello *et al.*, 2007; Grab *et al.*, 1998; Guo *et al.*, 1995; Probert & Johnson, 1998; Zambrano *et al.*, 2004). Several borrelial outer-surface proteins have been identified that exhibit affinities for some of those host components (Fischer *et al.*, 2006; Guo *et al.*, 1995; Probert & Johnson, 1998).

All examined infectious Lyme disease spirochaete isolates naturally contain between 6 and 10 distinct, but homologous, DNA elements called cp32s (Casjens *et al.*, 2006; Stevenson *et al.*, 2001, 2006). Most cp32 elements are circular episomes approximately 32 kb in size. However, some naturally occurring mutant cp32 family members have been identified, such as the lp56 element of *B. burgdorferi* type strain B31, which consists of an entire cp32

integrated into an unrelated linear replicon (Casjens *et al.*, 2000). Each cp32 contains a mono- or bicistronic *erp* locus, which generally varies in sequence between the different cp32s carried by an individual bacterium (Stevenson *et al.*, 2001, 2006). Moreover, different bacterial strains often contain unique *erp* genes. Erp proteins are surface-exposed outer-membrane lipoproteins that are highly expressed during mammalian infection but generally repressed during colonization of the tick vector (Das *et al.*, 1997; El-Hage *et al.*, 2001; Gilmore *et al.*, 2001; Hefty *et al.*, 2002; Lam *et al.*, 1994; Liang *et al.*, 2002; McDowell *et al.*, 2001; Miller *et al.*, 2003, 2005; Miller & Stevenson, 2006; Miller *et al.*, 2006; Skare *et al.*, 1999).

To date, functions have been ascribed to only a few borrelial Erp proteins, but are consistent with expression of this family of proteins during vertebrate infection. Several closely related (>85% amino acid sequence identities) Erp proteins bind both mammalian factor H and plasminogen/plasmin (Alitalo *et al.*, 2002; Brissette *et al.*, 2009; Hellwege *et al.*, 2001; Kraiczy *et al.*, 2003, 2004; Metts *et al.*, 2003; Stevenson *et al.*, 2002). One member of that group, ErpP (also called BbCRASP-3), binds another, distinct human serum protein, factor H-related protein 1 (FHR-1) (Haupt *et al.*, 2007). At least one other Erp protein adheres to an unknown component(s) of murine endothelium (Antonara *et al.*, 2007).

We now present results of the first studies on interactions between *B. burgdorferi* and mammalian laminin, a major component of vertebrate basement membranes and many other connective tissues (Colognato & Yurchenco, 2000; Freinkel & Woodley, 2001; Sasaki *et al.*, 2004). Such interactions may facilitate migration of *B. burgdorferi* through extracellular matrices and/or assist in long-term

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Abbreviation: ECM, extracellular matrix.

A supplementary sequence alignment is available with the online version of this paper.

colonization of laminin-containing host tissues. *B. burgdorferi* was found to produce several membrane-associated laminin-binding proteins, including the ErpX outer-membrane lipoprotein.

METHODS

Bacteria. *B. burgdorferi* strain B31 is the species type strain (Johnson *et al.*, 1984). The complete genome sequence of a non-clonal subculture of strain B31, called B31-MI, has been determined (Casjens *et al.*, 2000; Fraser *et al.*, 1997). A total of 10 distinct *erp*-bearing cp32 family members have been identified in strain B31, as follows: three separate, identical copies of *erpAB* on cp32-1, cp32-5 and cp32-8 (formerly known as *erpAB*, *erpIJ* and *erpNO*, respectively); *erpCD* on cp32-2; *erpG* on cp32-3; *erpHY* on cp32-4; *erpK* on cp32-6; *erpLM* on cp32-7; *erpPQ* on cp32-9; and *erpX* on lp56 (Casjens *et al.*, 1997, 2000; Stevenson *et al.*, 1996). The present studies all used an infectious clone derived from the sequenced culture, named B31-MI-16, that contains all the naturally occurring DNA elements of B31-MI (Miller *et al.*, 2003).

To date, the complete *erp* contents of four additional strains of Lyme disease spirochaetes have been determined: *B. burgdorferi* strains N40, 297, Sh-2-82 and BL206 (Akins *et al.*, 1999; Stevenson & Miller, 2003; and our unpublished results). Strains B31 and BL206 are nearly identical to each other, and have been classified as members of intergenic spacer type (IGS) group 1, as determined by sequence analysis of the 16S–23S rRNA gene spacer region (Bunikis *et al.*, 2004; Terekhova *et al.*, 2006; Wang *et al.*, 2001; and our unpublished results). Strains 297 and Sh-2-82 are also nearly identical to each other, and are members of IGS group 2 (Akins *et al.*, 1999; Bunikis *et al.*, 2004; Stevenson & Miller, 2003). Strain N40 is largely distinct genetically from the other analysed strains, and is a member of IGS group 9 (Bunikis *et al.*, 2004; Stevenson & Miller, 2003). Sequences of those strains' *erp* genes and their encoded proteins were compared with those of strain B31 to identify similarities using CLUSTAL_X (Thompson *et al.*, 1997) with default parameter settings.

B. burgdorferi was cultured in Barbour–Stoenner–Kelly II medium (BSK-II) at 34 °C (Zückert, 2007). Cultures (100 ml) used for bacterial cell lysates were harvested at mid-exponential phase (approx. 10^7 bacteria ml^{-1}) and fractionated using Triton X-114 (Pryde, 1986). This method yields three fractions from the bacteria: a detergent-soluble fraction primarily consisting of outer-membrane components, an aqueous fraction consisting of periplasmic components, and a pellet that primarily consists of inner-membrane and cytoplasmic components (Cunningham *et al.*, 1988; El-Hage *et al.*, 2001; Radolf *et al.*, 1988). The detergent-soluble, outer-membrane-enriched fraction was used for adhesin analyses.

Interactions between intact *B. burgdorferi* and laminin. Glass microscope slides were washed with deionized water, then coated by overnight incubation with $5 \mu\text{g ml}^{-1}$ Engelbreth–Holm–Swarm (EHS) mouse sarcoma laminin (Sigma-Aldrich) in PBS. Mice and other rodents are natural reservoir hosts of *B. burgdorferi*, particularly so in the north-eastern USA, where strain B31 was isolated (Lane *et al.*, 1991). Control slides were coated similarly with bovine serum albumin (BSA). The following day, slides were washed three times with PBS, then blocked by incubation with 3% (w/v) BSA for 2 h at room temperature. Cultured B31-MI-16 (10^7 bacteria ml^{-1} , mid-exponential phase) were harvested by centrifugation, washed once with PBS and resuspended in PBS to the original volume. Slides were covered with suspended bacteria, incubated at 37 °C for 2 h, then gently washed 10 times with PBS. Bacteria were visualized by darkfield microscopy. Numbers of adherent bacteria observed in ten 200 × fields per slide were counted.

Fig. 1. Adherence of intact *B. burgdorferi* to laminin. Bars indicate 1 standard deviation from the mean. Asterisks indicate statistically significant differences between adherence to laminin and BSA ($P < 0.001$). (A) Microscopical enumeration of bacteria adhering to slides coated with laminin or BSA at initial concentrations of $5 \mu\text{g ml}^{-1}$. (B) Results of ELISA-based studies of live bacteria binding to fixed laminin, means of 30 assays. Assays without added bacteria served as negative controls.

B. burgdorferi–laminin interactions were quantified by ELISA as follows. Maxisorp 96-well plates (Nalge-Nunc) were coated overnight with $10 \mu\text{g ml}^{-1}$ EHS laminin (Sigma-Aldrich) in 50 mM NaHCO_3 (pH 9.6) at 4 °C (Stevenson *et al.*, 2007). Plates were brought to room temperature and washed once with PBS + 0.5% (v/v) Tween 20 (PBS-T). Wells were blocked for 2 h at room temperature with 2% (w/v) BSA in PBS, then washed three times with PBS-T. Cultured B31-MI-16 (10^7 bacteria ml^{-1} , mid-exponential phase) were harvested, washed once with PBS and resuspended to the original volume. Aliquots (100 μl) of bacteria were added to each well and incubated for 2 h at 37 °C. Wells were washed three times with PBS-T, then incubated for 1 h at room temperature with rabbit antiserum raised against a membrane-enriched extract of cultured *B. burgdorferi* strain B31, diluted 1:10 000 in PBS (Miller *et al.*, 2003). Plates were washed three times with PBS-T, then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated anti-rabbit IgG (GE Healthcare), diluted 1:10 000. Wells were again washed three times with PBS-T, 100 μl per well tetramethylbenzidine substrate (Sigma-Aldrich) was added, then reactions were stopped by addition of 1 M H_2SO_4 , 100 μl per well. Absorbance was read at 450 nm using a Spectramax plate reader using SoftMax Pro (Molecular Devices).

The effects of soluble recombinant ErpX and mutant proteins upon laminin binding by live *B. burgdorferi* were examined by ELISA as described above, with one additional step. After washing the laminin-coated wells, a 100 μl aliquot of a recombinant protein or BSA (initial concentration $10 \mu\text{g ml}^{-1}$) was added to each well, followed by incubation at 37 °C for 30 min. Bacteria were then added directly to each well. Each assay was replicated at least six times.

Statistical analyses were performed using Student's *t*-test assuming unequal variances.

Recombinant proteins. Most of the polyhistidine-tagged, full-length recombinant Erp proteins used in this work have been described earlier (El-Hage & Stevenson, 2002; Stevenson *et al.*, 1998). All recombinant proteins contained amino-terminal tags, with the Erp segment beginning with that protein's first amino acid following

the conserved cysteine lipidation site. Expression plasmids for recombinant ErpA, ErpC, ErpL, ErpP, ErpQ, ErpY and ErpX were based on pET15b (Novagen), plasmids producing recombinant ErpG, ErpK and ErpM were based on pProEX-1 (Life Technologies), and that for recombinant ErpB was based on pET100 (Invitrogen). Two additional constructs, encoding carboxy-terminally truncated recombinant ErpX proteins, were produced from B31-MI-16 total genomic DNA using oligonucleotide primer pairs 5'-CACCAAGATTGATGCAACTGGTAAAG-3' plus 5'-AATTCTTAAATTTAGAA-TTG-3' or 5'-CACCAAGATTGATGCAACTGGTAAAG-3' plus 5'-TTCTTAAACCTTTCTAATCCTG-3', and cloned into pET200 (Invitrogen). Further constructs encoding additionally amino- or carboxy-terminally truncated recombinant ErpX proteins were produced from those pET200-based clones, using sequence-overlap extension PCR mutagenesis (Ho *et al.*, 1989) to either delete sections of the *erpX* open reading frame or introduce premature stop codons. A plasmid encoding recombinant Erp26 was produced from a plasmid clone of the strain N40 locus (Stevenson & Miller, 2003), using pET200 as its basis. Inserts of all recombinant plasmids were entirely sequenced on both strands to ensure that no undesired mutations had occurred during PCR or cloning procedures.

Recombinant proteins were expressed in *Escherichia coli* Rosetta(DE3)pLysS (Novagen), upon induction with IPTG. Induced *E. coli* were harvested, lysed by sonication, and debris cleared by centrifugation. Recombinant proteins were purified from cleared lysates using MagneHis nickel-conjugated magnetic beads (Promega). Concentrations of protein preparations were determined by bicinchoninic acid assay as compared with defined stock concentrations of BSA (Pierce).

Ligand-affinity blot analysis of laminin binding. Immediately prior to use, all apparatus used in these analyses was thoroughly washed with detergent and rinsed with deionized water, since laminin from human skin was found to be a common contaminant of laboratory equipment. Commercially obtained protein molecular mass standards were also found to be frequently contaminated with laminin or substances able to bind laminin, so at least one empty lane was left between the molecular mass standards and the proteins being examined, to avoid blurring of blot signals. BSA was used to block membranes after transfer, since the more commonly used blocking agent non-fat dried milk contains laminin, which obscures results of these analyses.

Recombinant proteins, Triton X-114 outer-membrane fractions, or control protein BSA were suspended in SDS gel loading buffer [125 mM Tris (pH 6.8), 20 % (v/v) glycerol, 4 % (w/v) SDS, 10 % (v/v) β -mercaptoethanol], and heated in a boiling water bath for approximately 1 min. An aliquot (approx. 1 μ g) of each protein was loaded into wells of polyacrylamide gels (without added SDS), then subjected to electrophoresis. Proteins were electrotransferred to nitrocellulose membranes, then blocked overnight at 4 °C with 5 % (w/v) BSA in Tris-buffered saline-Tween 20 [TBS-T; 20 mM Tris (pH 7.5), 150 mM NaCl, 0.05 % (v/v) Tween 20]. Membranes were next washed with TBS-T, and incubated for 1 h at room temperature in 13 μ g ml⁻¹ EHS laminin (Sigma-Aldrich) in TBS-T. After extensive washing with TBS-T, membranes were incubated for 1 h at room temperature in affinity-isolated rabbit anti-EHS laminin polyclonal antiserum (Sigma-Aldrich), diluted 1:2500 in TBS-T. Membranes were again washed with TBS-T, then incubated for 1 h at room temperature with horseradish-peroxidase-conjugated donkey anti-rabbit Ig antibody (GE Healthcare), diluted 1:5000 in TBS-T. After a final series of washes with TBS-T, bound antibodies were detected using SuperSignal West Pico enhanced chemiluminescence substrate (Pierce). Each recombinant protein was assayed at least twice.

Modelling analyses. Predictions of disorder within proteins were determined using Predictor of Naturally Disordered Regions (PONDR,

www.pondr.com) using VL-XT. Access to PONDR was provided by Molecular Kinetics (6201 La Pas Trail - Ste 160, Indianapolis, IN 46268, USA; +1 317 280-8737; e-mail: main@molecularkinetics.com). VL-XT is copyright © 1999 by the WSU Research Foundation, all rights reserved, and PONDR is copyright © 2004 by Molecular Kinetics, all rights reserved. Primary amino acid sequences of *B. burgdorferi* proteins and truncated protein fragments were compared with all known sequences in the non-redundant protein sequence database of GenBank using BLASTP and PSI-BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). Protein folding probabilities were determined using Protein Homology/analogy Recognition Engine (PHYRE) (<http://www.sbg.bio.ic.ac.uk/~phyre>).

RESULTS

B. burgdorferi adheres to laminin

As noted in the Introduction, Lyme disease spirochaetes are frequently found associated with host ECM during vertebrate infection. Since laminin is a major component of basement membranes and other mammalian ECMs (Freinkel & Woodley, 2001; Sasaki *et al.*, 2004), the ability of *B. burgdorferi* to adhere to laminin was examined in detail. Intact, live *B. burgdorferi* were observed to adhere to laminin-coated microscope slides in significantly greater numbers than to BSA-coated slides (Fig. 1A). Spirochaete-laminin interactions were further quantified through use of an ELISA-based method, again demonstrating that *B. burgdorferi* exhibits a marked affinity for laminin (Fig. 1B).

The specificity of *B. burgdorferi*-laminin interactions was further analysed by ligand-affinity blot analyses using purified laminin and an outer-membrane fraction of *B. burgdorferi*. This technique indicated that cultured *B. burgdorferi* strain B31 produced three proteins with detectable affinities for laminin, having approximate molecular masses of 40 and 50 kDa (Fig. 2).

ErpX binds mammalian laminin

Studies by our laboratories and others have demonstrated that all *B. burgdorferi* *erp* genes so far examined are expressed during mammalian infection (our unpublished results; Crother *et al.*, 2004; Liang *et al.*, 2002; Miller *et al.*, 2005; Miller & Stevenson, 2006). Noting that the laminin-binding outer-membrane proteins detected in Fig. 2 are of sizes similar to some Erp proteins (Casjens *et al.*, 2000; Stevenson *et al.*, 1998, 2006), we hypothesized that one or more of the borrelial laminin-binding proteins may be an Erp protein. To explore that possibility, recombinant Erp proteins were produced from all the *erp* gene sequences carried by *B. burgdorferi* strain B31. Equal amounts of each protein were then subjected to ligand-affinity blot analyses to determine their abilities to bind laminin. BSA was also included, as a negative control to demonstrate that ligand binding was not due to non-specific protein-protein interactions. As described below, most of the recombinant Erp proteins did not bind laminin, and therefore also served as controls for specificity of laminin binding.

Fig. 2. Ligand-affinity blot analyses of laminin binding to components of outer-membrane-enriched Triton X-114 extract of cultured *B. burgdorferi* B31-MI-16. Arrows to the right indicate bacterial proteins that bound laminin. Positions of molecular mass standards are indicated to the left.

Of the strain B31-derived Erp proteins, only recombinant ErpX bound laminin with a detectable affinity (Fig. 3A). Native, lipidated ErpX migrates in SDS-PAGE with an apparent molecular mass of approximately 45–50 kDa (El-Hage *et al.*, 2001; Stevenson *et al.*, 1998), and was probably the larger laminin-binding protein observed in Fig. 2. Several of the other tested recombinant Erp proteins (ErpA, ErpC, ErpL, ErpP, ErpQ, ErpY) were produced using the same cloning vector as was ErpX, and all included the same amino-terminal tag, so the inability of those other recombinant proteins to bind laminin demonstrated that laminin binding by recombinant ErpX was not due to its polyhistidine tag. The *erpX* gene sequence is distinct from all the other *erp* genes of *B. burgdorferi* strain B31

Fig. 3. Ligand-affinity blot analyses of laminin binding by polyhistidine-tagged recombinant Erp proteins. (A) Recombinant Erp proteins of *B. burgdorferi* strain B31. BSA was included as a control for non-specific protein–laminin interactions. (B) Recombinant ErpX from strain B31 and Erp26 from strain N40. Positions of molecular mass standards are indicated between the two panels.

(Stevenson *et al.*, 2006). Of the strain B31 Erp proteins, ErpX shares the highest degree of similarity with ErpQ protein (see Supplementary Fig. S1, available with the online version of this paper), which did not detectably bind laminin, indicating that the laminin-binding region(s) of ErpX is distinct from any sequence found in ErpQ.

The complete *erp* gene sequences of several additional strains of the Lyme disease spirochaete have been determined. Of all the known borrelial *erp* genes, the strain N40 *erp26* gene shares the highest degree of similarity with the strain B31 *erpX* gene (Fig. S1 and Stevenson & Miller, 2003; Stevenson *et al.*, 2006). Due to that similarity, recombinant N40 Erp26 protein was produced and assayed for ability to bind laminin. However, recombinant Erp26 was not able to bind laminin at detectable levels (Fig. 3B). Thus, the laminin-binding domain(s) of ErpX is also distinct from any sequence within Erp26. Studies described below determined that the laminin-binding domain of ErpX consists of sequences unlike any found in either the N40 Erp26 or B31 ErpQ proteins, consistent with the results of these ligand-affinity analyses.

Mapping the laminin-binding domain of ErpX

Truncated variants of ErpX were produced to narrow down the region of that protein involved with binding of laminin. A distinctive characteristic of ErpX is a fivefold repeat of the 5 amino acid sequence aspartate-alanine-threonine-glycine-lysine near the protein's amino terminus (Fig. 4A and Fig. S1). However, truncated variants of ErpX lacking the repeated amino acid sequence, such as rErpX3 and rErpX5, did not show any appreciable differences in laminin binding, indicating that the amino acid repeat region does not play a direct role in adhesion to laminin (Figs 4A and 5A). Additional amino- and carboxy-terminally truncated variants of ErpX were produced and assessed for ability to bind laminin. Truncated proteins rErpX3 through rErpX12 all bound laminin with the same apparent affinities as did the full-length, wild-type rErpX protein (Figs 4A and 5A, and data not shown).

Loss of ligand binding was observed upon truncation of ErpX to the 112 residue rErpX16 (Figs 4 and 5B). Recombinant protein rErpX14, consisting of 90 amino acids nested within rErpX16, was similarly unable to bind laminin. However, protein rErpX15, which contains only 84 amino acids, retained laminin-binding activity. Since the sequences of rErpX15 and rErpX16 overlap (Fig. 4A), these results indicate that the carboxy-terminal 32 amino acids of rErpX15 include residues critical for binding of laminin.

Following the result of studies with rErpX15 and rErpX16, protein rErpX17 was produced, consisting of the entire ErpX sequence except for 55 central amino acids (Fig. 4). Consistent with the ability of rErpX15 to bind laminin and the inability of rErpX14 and rErpX16 to bind that ligand, rErpX17 did not bind laminin at detectable levels (Fig. 5C).

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rErpX      KIDATGKDATGKDATGKDATGKDAEQNIGKVGQFLEKILDPVKDIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX3     KIDATGKNABQNIKGKVGQFLEKILDPVKDIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX5     QNIKGKVGQFLEKILDPVKDIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX12    VKDKIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX13    VNNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX15    VKDKIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX16    VKDKIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX14    VKDKIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED
rErpX17    KIDATGKDATGKDATGKDATGKDAEQNIGKVGQFLEKILDPVKDIASNGPIADELAKKLQEEKVNNGEEENDKAVFLGEESKEDEEENEQAVNLEEKNAEED

rErpX      KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKKQEQEKKRKRQEQKERRAKNKIKKLADKIDEISWNIDGIESQTSVKPKAVIDKITGPV
rErpX3     KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKKQEQEKKRKRQEQKERRAKNKIKKLADKIDEISWNIDGIESQTSVKPKAVIDKITGPV
rErpX12    KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKKQEQEKKRKRQEQKERRAKNKIKKLADKIDE
rErpX13    KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKKQEQEKKRKRQEQKERRAKNKIKKLADKIDE
rErpX15    KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKKQEQEKKRKRQEQKERRAKNKIKKLADKIDE
rErpX16    KKVVNLEEKELEVKKETEDEDKKEIEKQKQVEKAQERKQREQEKKRKK
rErpX14    KKVVNLEEKELEVKKETEDEDKKEIEK
rErpX17    KKVVNLEEKELEVKKETEDEDKKEIEK.....ISWNIDGIESQTSVKPKAVIDKITGPV

rErpX      YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKFEEIKGYISYSQ
rErpX3     YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX5     YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX12    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX13    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX15    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX16    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX14    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKF
rErpX17    YDYFTDDNKKAIYKTWGDLEDEEGEGLGKLLKELSDTRDELRTKLNKDNKKYAHENEPPLKENVDVSEIKEDLEKVKSGLEKVKYKLDNSKFEEIRGYISYSQ

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B

Fig. 4. (A) Amino acid sequences of recombinant ErpX (rErpX) and the truncated derivatives of ErpX used in these studies. The 55 amino acids deleted from rErpX17 are indicated by dots. (B) Predicted order/disorder of wild-type ErpX. A PONDR score above 0.5 predicts protein disorder; a score below 0.5 predicts order. The laminin-binding region in the centre of ErpX, as identified through use of variant proteins rErpX15, rErpX16 and rErpX17, is predicted to be extremely disordered in the absence of bound ligand.

Full-length rErpX and several fragments were analysed for abilities to interfere with adhesion of *B. burgdorferi* to laminin. Consistent with the above-described ligand-affinity blot analyses, rErpX, rErpX-12, rErpX-13 and rErpX-15 all reduced *B. burgdorferi* adherence to laminin

by 60–70 % (Fig. 6). Protein rErpX-17 inhibited binding by approximately 20 %, suggesting that this protein retains some, otherwise undetectable affinity for laminin. Addition of the control protein, BSA, did not influence laminin-binding by the spirochaetes.

Fig. 5. Ligand-affinity blot analysis of laminin binding by recombinant ErpX (rErpX) and truncated derivatives thereof. (A) Full-length ErpX and three of the examined truncations. The rightmost lane contained an equal molar content of BSA, to serve as a control for non-specific laminin binding. (B) The upper panel shows results of laminin-binding analysis of truncated proteins rErpX12, rErpX13, rErpX15, rErpX16 and rErpX14, and the lower panel shows the same amounts of each protein in a gel stained with Coomassie brilliant blue. Note that even though relatively less rErpX15 appears to have been loaded onto this gel, the laminin-binding signal for that protein was comparable to those from ErpX12 and rErpX13. (C) The upper panel illustrates results of laminin-binding analysis of proteins rErpX3 and rErpX17, and the lower panel shows the proteins stained with Coomassie brilliant blue. Migration positions of molecular mass standards are indicated on the left of each panel. Highly charged proteins such as ErpX often exhibit aberrant SDS-PAGE mobilities (Stevenson *et al.*, 1998): note that rErpX-5 migrated at a rate suggestive of a molecular mass greater than rErpX-3, even though rErpX-5 is actually the smaller protein.

As are all Erp proteins, ErpX is a highly charged molecule, with the full-length, mature protein containing 18% glutamate, 9% aspartate and 19% lysine residues and having a predicted pI of 4.9. The central, laminin-binding region defined by the deletion within rErpX17 has a predicted pI of 11.1 and consists of 20% glutamate, 4% aspartate, 29% lysine, 18% glutamine and 15% arginine residues (Fig. 4A). Modelling analyses predicted that this central region is largely disordered (Fig. 4B). The flanking sections of the full-length ErpX protein possess more

structured natures, which could serve to stabilize or orient the protein's middle, laminin-binding region. Neither BLASTP nor PSI-BLAST detected similarities between the laminin-binding region of ErpX and any previously identified predicted protein. Likewise, PHYRE analyses did not predict either the central region or the full-length ErpX to have similarities with any known protein structure. ErpX appears to represent a novel type of bacterial laminin adhesin.

DISCUSSION

Lyme disease spirochaetes reside extracellularly during vertebrate infection. Several previous studies found that *B. burgdorferi* interacts with constituents of host ECM and cell-surface components. The present studies appear to be the first to demonstrate that *B. burgdorferi* adheres to laminin. Analyses of outer-membrane-enriched fractions of the cultured *B. burgdorferi* type strain B31 indicated that it produces three proteins with detectable affinities for laminin. We determined that the surface-exposed lipoprotein ErpX is a laminin-binding protein, and mapped its ligand-binding site to a short, unstructured region in the centre of the protein. Serological, RNA array and gene-specific Q-RT-PCR analyses have all indicated that *B. burgdorferi* produces ErpX during mammalian infection (our unpublished results; Liang *et al.*, 2002; Skare *et al.*, 1999; Stevenson *et al.*, 1998). Thus, ErpX is appropriately poised on the borrelial outer surface to interact with host laminin, and we hypothesize that this bacterial protein contributes to invasion and/or long-term colonization of host tissues. Prior studies indicated that *B. burgdorferi* strains which lack lp56 (and are therefore *erpX* deficient)

Fig. 6. Effects of recombinant proteins on adherence of *B. burgdorferi* to laminin, as measured by ELISA. Results for each protein are illustrated relative to control experiments lacking added protein. All added ErpX derivatives significantly affected adherence to laminin: rErpX-17 ($P=0.0009$), and rErpX, rErpX-12, rErpX-13 and rErpX-15 (all $P<0.0001$). Bb, *B. burgdorferi*.

are still able to infect mice (Purser & Norris, 2000), which may be due to binding of additional host ECM components by other borrelial surface proteins or to the redundancy of laminin-binding proteins produced by *B. burgdorferi* (see Fig. 2). Preliminary analyses of the other laminin adhesins with apparent molecular masses of ~40 kDa suggest them to be members of the chromosomally encoded Bmp (p39) protein family (our unpublished results). Complete characterization of the other laminin-binding proteins is under way, with the goals of defining their mechanisms of ligand adhesion and producing mutant bacteria deficient in all laminin adhesins for mouse and tick infection studies.

There are no apparent similarities between ErpX and the known laminin-binding proteins of the spirochaetes *Leptospira interrogans* (Barbosa *et al.*, 2006; Stevenson *et al.*, 2007), *Treponema pallidum* (Cameron, 2003; Cameron *et al.*, 2005) or *T. denticola* (Edwards *et al.*, 2005), nor of any other organism, suggesting that ErpX adhesion to laminin may occur through a novel mechanism. The predicted disordered nature of the laminin-binding region of ErpX is reminiscent of the fibronectin-binding domain of the *B. burgdorferi* BBK32/Fbp/p47 protein, in which the binding site consists of a disordered sequence of amino acids which moulds itself around the ligand (Kim *et al.*, 2004).

One striking feature of ErpX is the fivefold reiterated 5 amino acid sequence near the amino terminus. Our studies demonstrated that this sequence is not directly involved with laminin binding. The directly repeated sequences of *erpX* presumably arose through either cross-fork slippage or recombination events (Lovett, 2004). It is significant that this direct repeat involves a multiple of 3 bp, such that the reiterations did not alter the reading frame, and indicating that this repeat element cannot be involved with phase variation as occurs in bacteria such as *Neisseria gonorrhoeae* or *Haemophilus influenzae*. We also observed that the *erpX* genes of *B. burgdorferi* strains B31 and BL206 are identical (our unpublished results). As strain BL206 was isolated from nature 16 years after strain B31, and must have infected numerous vertebrate and tick hosts since the two strains shared a common ancestor, this direct repeat mutation appears to be highly stable. A possible function for the repeated amino-terminal motif of ErpX is suggested from studies of other bacterial adhesins: repeated amino acid sequences near the membrane anchor can provide distance between adhesive domains and the bacterial cell surface, and the greater the number of repeats, the further the adhesin extends into the environment (Patti *et al.*, 1994; van Belkum, 1999).

In conclusion, we have determined that the *B. burgdorferi* outer-surface lipoprotein ErpX binds mammalian laminin, through a protein motif not previously recognized in other bacterial laminin adhesins. No other Erp proteins of strain B31 bound laminin, nor did the Erp26 protein of strain N40, and the functions of most of those proteins remain a mystery. However, the Lyme disease spirochaete produces

at least two other laminin-binding proteins, neither of which is an Erp protein. It is possible that dissimilarities among the repertoires of Erp and other proteins produced by different *B. burgdorferi* strains may play roles in the variations in infectivity observed among isolates (Balmelli & Piffaretti, 1995; Liveris *et al.*, 2002; Seinost *et al.*, 1999; Shih *et al.*, 1992; Terekhova *et al.*, 2006; van Dam *et al.*, 1993; Wang *et al.*, 2002). Similarly, differences in amino acid sequences and modifications to host ECM components such as laminin may contribute to the variations among borreliae in their abilities to efficiently infect different host species (Kurtenbach *et al.*, 2006). Further characterization of borrelial adhesins and examination of interactions between these bacterial proteins and host tissues will address those hypotheses, providing insight into the pathogenic properties of Lyme disease spirochaetes. Such information could direct development of therapeutic treatments that block adhesin-ECM interactions or disrupt production of adhesins associated with mammalian infection.

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