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Identification of two putative rickettsial adhesins by proteomic analysis

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Abstract

The rickettsial membrane proteins that promote their uptake by eukaryotic host cells are unknown. To identify rickettsial ligand(s) that bind host cell surface proteins, biotinylated epithelial cells were used to probe a nitrocellulose membrane containing rickettsial extracts separated by SDS–PAGE. This overlay assay revealed that two close rickettsial ligands of approximately 32–30 kDa were recognized by host cells. Both proteins were identified using high resolution 2D-PAGE coupled with mass spectrometry analysis. One protein was identified as the C-terminal extremity of rOmpB called the β-peptide. The second interacting protein was identified as a protein of unknown function encoded by RC1281 and RP828 in *Rickettsia conorii* and in *Rickettsia prowazekii,* respectively, that shares strong similarities with other bacterial adhesins. Both proteins are highly conserved within the *Rickettsia* genus and might play a critical role in their pathogenicity. These data may have important implications for the development of future vaccines against rickettsial infections.

Keywords: Rickettsiae; Adhesin; rOmpB

1. Introduction

Rickettsia species are small, Gram-negative bacilli that are obligate intracellular parasites of eukaryotic cells. This genus consists of two antigenically defined groups: the spotted fever group (SFG) and the typhus group (TG), which are respectively responsible for spotted fevers and typhus [36]. Electron microscopy studies show that initial contact between these bacteria and endothelial cells results in a dramatic alteration of the cell surface, with the formation of numerous microvillar extensions associated with membrane ruffling [42]. However, the molecular mechanisms through which rickettsiae attach to and induce their internalization by host cells are still largely unknown [47].

The attachment of microorganisms to a biological surface is a complex process involving both non-specific and specific events. Non-specific attachment can be mediated by electrostatic forces, lipophilic and hydrophobic interactions. Specific "lock and key"-type interactions between lectin-like bacterial binding proteins (adhesins) and complex glycoprotein receptors on host tissues have also been described [15,48]. Adhesin proteins can be located at the distal tip of non-flagellar surface appendages known as pili or fibrillae [21]. Afimbrial adhesins, which include most other adherence molecules, have also been reported. Characterized afimbrial adhesins include BabA adhesin from *Helicobacter pylori* [18], outer-membrane proteins (Vomp) from *Bartonella quintana* [51], filamentous hemagglutinin and pertactin from *Bordetella pertussis* [7], opacity proteins (Opas) from *Neisseria* and invasin from enteropathogenic *Yersinia* [19]. Another major constituent of the outer membrane of Gram-negative bacteria that plays a critical role in the interface between microorganisms and the environment is lipopolysaccharide (LPS). Its role as an adhesin has been

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suggested for several bacteria including *Actinobacillus pleuropneumoniae*, *Campylobacter jejuni*, *Helicobacter pylori* and *Neisseria gonorrhoeae* [20].

Rickettsiae express two major surface proteins, termed rickettsial outer membrane proteins A (rOmpA) and B (rOmpB) [2,24]. Both proteins are present in SFG rickettsiae, but only rOmpB is present in TG [26,50]. As a consequence of a gene degradation of *rompA* [5,37], rOmpA is exclusive for SFG. It is well established that rOmpA and rOmpB are immunodominant antigens containing protective epitopes. Protection afforded by the recombinant truncated rOmpA protein against rickettsial infection has been demonstrated in a *R. rickettsii*–mouse model [28,41], and in a *R. conorii*–guinea pig model [46]. Vaccine efficacy of plasmid DNA encoding recombinant rOmpA has also been reported [9,41]. Protection against lethal infection of C3H/HeN SCID mice by *R. conorii* was induced by mAbs against rOmpA and rOmpB [45]. Similarly, protection of mice against lethal challenge with *R. typhi* was induced by immunization with native *R. typhi* rOmpB protein [6,10,45]. In contrast, passive immunization with antibodies against LPS does not protect mice from infection with viable rickettsiae [1,14,45].

With the exception of *R. akari* (the agent of rickettsialpox) which infects macrophages, rickettsiae target primarily endothelial cells [36]. Several lines of evidence suggest that rOmpA and rOmpB are involved in adherence of these microorganisms to eukaryotic cells [26]. Two additional outer membrane proteins of 31 and 29.5 kDa are also implicated in adherence of *R. prowazekii* [11]. The 31 kDa protein is thought to correspond to the cleavable C-terminal domain of rOmpB [17], whereas the 29.5 kDa protein is the preprotein translocase SecA subunit [12].

Despite numerous efforts [35,39,43], genetic manipulation of *Rickettsia* remained impossible for years [49]. While recent progress in the field of the genetic transformation of *Rickettsia* is hopeful [33], it is still difficult to specifically knock out a rickettsial gene and to firmly demonstrate the biological role of the corresponding encoded protein. Recognition of and binding to the host cell is a key step in the pathogenesis of many virulent bacterial strains, and identification of the molecular basis of rickettsial attachment to host cells remains an important objective. This is particularly true when considering the fact that these strictly intracellular bacteria, which are considered as a threat for use as bioterrorism agents [4], must enter host cells to replicate and survive.

The last few years have seen a dramatic increase in the number of sequenced bacterial genomes, accompanied by a rapid acceleration in the development of new bioinformatics tools. Large-scale DNA sequencing has also stimulated the development of technological changes in proteomics, namely two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) coupled with mass spectrometry which allows identification of proteins in a complex mixture. In this work, we used a global proteomic approach to identify rickettsial ligands involved in bacteria-host cell interactions. This study, which was carried out on both *R. conorii* (the agent of Mediterranean spotted fever) and *R. prowazekii* (the agent of epidemic typhus), led to identification of 2 putative rickettsial adhesins recognized by host cells.

2. Materials and methods

2.1. Rickettsiae and cells

R. conorii Seven (Malish), ATCC VR-610^T and *R. prowazekii* BreinL, ATCC VR-142^T were propagated on a confluent monolayer of African green monkey kidney cells (Vero cells, ATCC C1587) [36] and purified on renographin gradient as previously reported [13]. Bacterial pellets were solubilized in Laemmli buffer and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 12% acrylamide gel [22].

2.2. Biotinylation of epithelial cell proteins

Vero cells grown to confluence in 125 cm^2 flasks were washed with phosphate buffered saline (PBS), detached with beads and centrifuged. The pellet was resuspended in PBS containing 10 mg/ml of EZ-link sulfo-NHS-LC-biotin (Pierce) and incubated 1 h at 37 °C. After washing, pellets were submitted to sonication and insoluble components removed by centrifugation (10 000 *g* for 15 min at 4 °C). Biotinylated proteins were stored at −80 °C.

2.3. Overlay assays

Rickettsial extracts separated in one dimension by SDS–PAGE (20 µg) or by two-dimensional (2D)-PAGE (200 µg) were transferred to nitrocellulose membranes (Amersham). Membranes were incubated for 1.5 h at 4 °C with biotinylated epithelial cells diluted 1:100 in phosphate buffer saline (PBS) supplemented with 0.02% Tween-20 and 0.5% milk (PBS–Tween–milk). After washing three times, the blots were incubated with peroxidase-labeled streptavidin (1:1000, Amersham) and biotinylated proteins detected by chemiluminescence (ECL, Amersham).

2.4. Rickettsial membrane preparation for 2D-electrophoresis

The *R. conorii* and *R. prowazekii* pellets purified as described above were resuspended in 5 ml of 5 mM Tris–HCl buffer (pH 7.6) and lysed by two passes through a constant cell disruption system (2 kbar). Cell debris and unbroken cells were removed by centrifugation at 5600 *g* for 20 min. Bacterial membranes present in the supernatant were pelleted by ultracentrifugation at 100 000 *g* for 2 h and washed in 5 mM Tris–HCl, pH 7.6 before storage at −80 °C.

2.5. 2-D electrophoresis and silver staining

Immobiline™ DryStrips (18 cm, pH 3–11 or 6–11 Amersham) were rehydrated overnight with 350 µl rehydration buffer [8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% (v/v) IPG buffer

(Amersham Biosciences)] containing 200 µg of membrane-enriched rickettsial proteins. Isoelectric focusing (IEF) was carried out according to the manufacturer's protocol (Multiphor II, Pharmacia). Before second dimension electrophoresis, strips were equilibrated twice in equilibration buffer [30% (v/v) glycerol, 2% (w/v) SDS, 6 M urea, 50 mM Tris–HCl, bromophenol blue, pH 8.8] supplemented with DTT (65 mM) and iodoacetamide (100 mM), respectively. Proteins were resolved by 10% SDS–PAGE (Ettan™ DALT, Amersham) and the gels stained with silver by a method compatible with mass spectrometry [40] and digitally scanned (ImageScanner, Amersham). Spots excised from the gel were stored at −20 °C until identification. The spectra of at least two separate samples for each protein were analyzed and compared for accuracy.

2.6. In-gel digestion and MALDI-TOF MS

Protein spots were excised from gels, destained, then subjected to in-gel digestion with trypsin (sequencing grade modified porcine trypsine; Promega, Madison, WI, USA) [40]. Tryptic peptides were extracted from gel by successive treatment with 30% acetonitrile/5% formic acid and 50% acetonitrile/5% formic acid. Extracts were pooled and dried in a Speedvac evaporator. Peptides were solubilized with MALDI matrix α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.3% trifluoroacetic acid (TFA), applied to the metal target, then analyzed by MALDI-TOF MS (Bruker Ultraflex spectrometer, Bruker Daltonics, Wissembourg, France). Mass spectra were internally calibrated using autolytic peptides from trypsin.

2.7. Database searching and data interpretation

The peptide mass data obtained by MALDI-TOF MS spectral analyses were analyzed using Mascot software available online (http://us.expasy.org/). Protein domain searches were carried out using SMART [23] (http://smart.embl-heidelberg.de/) and NCBI-Conserved domain search programs [27] (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

2.8. Sequence alignment

The multiple sequence alignment of RC1281 was constructed using 3DCoffee [30] (http://igs-server.cnrs-mrs.fr/Tcoffee/tcoffee_cgi/index.cgi) by feeding the structural information of *Neisseria meningitidis* surface protein A (PDB: 1P4T), exhibiting significant sequence similarity (E-value = 2×10^{-12}) to the COG family (COG3637) of opacity proteins and related surface antigens. ESPript was used to generate the alignment figure [16] (http://prodes.toulouse.inra.fr/ESPript/ESPript/).

3. Results

3.1. Rickettsial ligands bind eukaryotic plasma membrane proteins

To identify rickettsial cell surface components that interact with eukaryotic cells, we probed separated crude rickettsial protein extracts with biotinylated Vero cell proteins in an overlay assay. As illustrated in Fig. 1, biotinylated epithelial proteins specifically recognize two rickettsial proteins with apparent MW of 30–32 kDa. A similar profile was obtained for both *R. conorii* and *R. prowazekii* lysates. When bacterial lysates were treated with trypsin before SDS–PAGE separation, this binding was abolished.

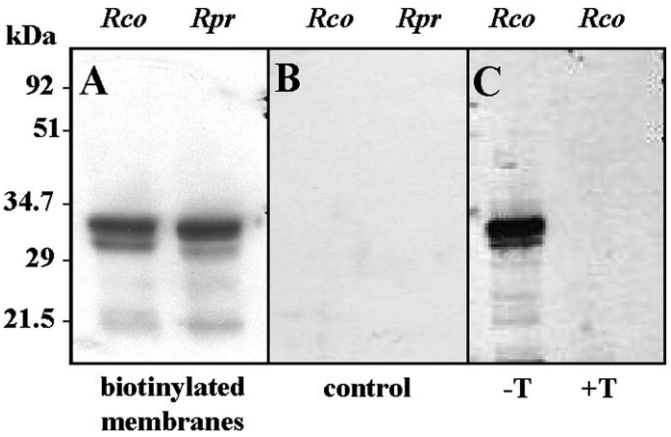

Fig. 1. Ligand overlay assay with epithelial cells. (A) Crude extracts of *R. conorii* or *R. prowazekii* cells separated by SDS–PAGE and transferred onto a nitrocellulose membrane, before incubation with epithelial cells previously biotinylated and sonicated. After washings, bound epithelial proteins were detected by western blot analysis with steptavine coupled with peroxidase. (B) Control experiments carried out without biotinylated eukaryotic cells. (C) Overlay assay performed on *R. conorii* pretreated (+T) or not (−T) with 0.25% trypsin for 30 min at 37 °C before SDS–PAGE. The positions of the molecular mass standards are shown on the left.

3.2. Characterization of rickettsial ligands recognized by Vero cells

To further characterize the 30–32 kDa interacting proteins, membrane-enriched extracts of both *R. conorii* and *R. prowazekii* were resolved by 2D-PAGE as previously described [38]. Separated proteins were blotted onto nitrocellulose membranes and overlaid with biotinylated Vero cells. Because the isoelectric points (pI) of the putative rickettsial ligands are unknown, experiments were initially performed using pH 3–11 NL strips. Following silver staining, around 1000 individual spots with MW between 20 to 100 kDa were detected (unpublished data). Of these proteins, 2 trains of spots with apparent MW of 32 and 30 kDa, respectively, were recognized by Vero cells and located within the basic pI range (data not shown). To increase the resolution within this pI range, the same experiment was performed using pH 6–11 strips. The resulting silver-stained 2D-gel obtained with the *R. conorii*-enriched membrane fraction is shown in Fig. 2. This high-resolution gel allowed subsequent identification by MALDI-TOF mass spectrometry of the protein spots excised from the gel. Proteins identified include malate dehydrogenase, RC0234-encoded heat-shock protease, PrsA precursor, trigger factor as well as proteins of unknown function such as RC0627 and RC0098. For all assigned proteins, identification was based on at least two and mostly three independent sets

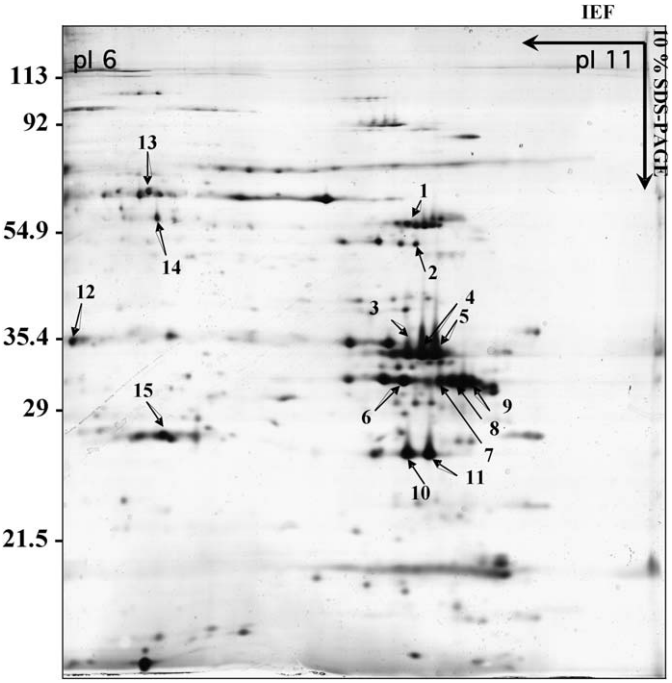

Fig. 2. 2D-PAGE profile *R. conorii* membrane-enriched extract. An *R. conorii* sample (200 µg) was separated using a pH 6–11 IPG strip in the first dimension followed by a 10% SDS–PAGE in the second dimension. The 2D gel was silver-stained. Proteins successfully identified are RC0627 (1), RC0098 (2), rOmpB (3–5), protein export PrsA precursor (6), RC1281 (7–9), RC1282 (10, 11), malate dehydrogenase (12), trigger factor (13), heat-shock protease (14) and thioredoxine peroxidase (15). Relative molecular masses (in kDa) are indicated on the left side.

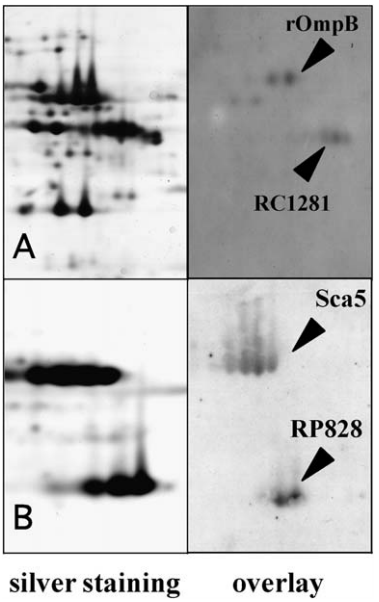

Fig. 3. (A) or *R. prowazekii* (B) membrane-enriched fractions were fractionated in a 2D-PAGE. Gels were stained for MS identification (left panels) or transferred to nitrocellulose membranes for overlay with epithelial proteins (right panels).

of data mapping to the same ORF. 2D-gel in conjugation with overlay assays also permitted to localize the spots interacting with Vero cell for both *R. conorii* and *R. prowazekii* extracts (Figs. 3A and 3B). Mass spectral analyses of the tryptic digest of the 32 kDa protein recognized by Vero cells was identified as rOmpB (RC1085) in *R. conorii* and its *R. prowazekii* ortholog, namely the cell surface antigen Sca5 (RP704). As illustrated in Fig. 3, several isoforms recognized by biotinylated eukaryotic cells matched this protein. In all cases, we obtained a low percent of coverage, ranging from 6 to 12%. This resulted from the difference between the observed molecular weight (MW) of the excised proteins (32 kDa) and the theoretical MW of *rompB*- and *sca5*-encoded proteins (168.3 and 169.8 kDa, respectively). As illustrated in Fig. 4, tryptic digestion of the 32 kDa *R. conorii* proteins yielded several peptides exclusively located in the C-terminal extremity of full rOmpB. It has been reported that, in the course of maturation, this molecule is cleaved into 120- and 32-kDa products, respectively [17]. It is the small cleavage product known as β-peptide that we identified as a potential rickettsial ligand. When considering only the β-peptide sequence starting from GDAE amino acids, the percent of coverage measured from tryptic digests was 32 and 34% for *R. conorii* and *R. prowazekii*, respectively.

The second train of *R. conorii* proteins recognized by Vero cells was identified as RC1281. Three distinct isoforms were detected, the percentage of coverage being 54% for all 3 proteins. Its homologous protein in *R. prowazekii*, namely RP828, was also recognized by biotinylated eukaryotic cells.

3.3. Bioinformatic analysis of identified rickettsial adhesins

R. conorii RC1085 (1655 aa) encodes the precursor of the outer membrane protein rOmpB. A protein domain search by SMART software [23] identified an autotransporter β-domain (PFAM: PF03797) at its C-terminal (position: 1368–1647 aa, with E-value $= 3.9 \times 10^{-36}$). In addition to the autotransporter domain, a signal peptide was also identified at the N-terminal of RC1085 (position: 1–20 aa), which could function to translocate the protein through the inner membrane. Comparative analysis of rickettsial cell surface antigens (*sca* family) within available rickettsial genomes showed that, among the 13 members of this family (*rompA*, *sca1–3*, *rompB*, *sca6–13*) [5,32], *rompB* was the unique conserved one. Moreover, sequences of orthologous rOmpB precursors of different rickettsiae exhibited the highest sequence conservation in the C-terminal domain.

R. conorii RC1281 exhibited significant BLAST scores (E-value $< 1 \times 10^{-50}$; NCBI non-redundant database search) with its orthologs in different *Rickettsia* species. It also exhibited significant sequence similarity to a paralogous ORF (RC1282, E-value $= 1 \times 10^{-28}$) of unknown function. These two tandemly encoded ORFs were conserved (including their positional links) in all 7 *Rickettsia* genomes sequenced thus far. These genomes are those of *R. prowazekii* [3], *R. conorii* [31], *R. typhi* [29], *R. rickettsii* (GenBank no. AADJ01000000), *R. sibirica* (GenBank no. NZ_AABW00000000), *R. akari* (GenBank no. AAFE00000000) and also that of *R. felis* [32]. RC1281 also exhibited rather weak sequence similarities to *Brucella suis* 1330 outer surface protein BR1689 (TrEMBL: Q8FZ14; E-value $= 0.45$), *Brucella melitensis*

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1       MAQKPNFLKKLISAGLVTASTATIVASFAGSAMGAAIQQNRTTNAVATTV
50      DGVGFDQTAVPANVAVPLNAVITAGVNKGITLNTPAGSFNGLFLNTANNL
150     DVTVREDTTLGFITNVVNNANHFNLMLNAGKTLTITGQGITNVQAAATKN
200     ANNVVAQVNNGAAIDNNDLQGVGRIDCGAAASTLVFNLANPTTQKAPLIL
250     GDNAVIVNGANGTLNVTNGFIKVSSKSFATVNVINIGDGQGIMFNTDADN
300     VNTLNLQANGATITFNGTDGTGRLVLLSKNAAATDFNVTGSLGGNLKGII
350     EFNTVAVNGQLKANAGANAAVIGTNNGAGRAAGFVVSVDNGKVATIDGQV
400     YAKDMVIQSANAVGQVNFRHIVDVGTDGTTAFKTAASKVAITQNSNFGTT
450     DFGNLAAQIIVPNTMTLNGNFTGDASNPGNTAGVITFDANGTLASASADA
500     NVAVTNNITAIEASGAGVVQLSGTHAAELRLGNAGSVFKLADGTVINGKV
550     NQTALVGGALAAGTITLDGSATITGDIGNAGGAAALQGITLANDATKTLT
600     LGGANIIGANGGTINFQANGGTIKLTSTQNNIVVDFDLAIATDQTGVVDA
650     SSLTNAQTLTINGKIGTVGANNKTLGQFNIGSSKTVLSDGDVAINELVIG
700     NNGAVQFAHNTYLITRTTNAAGQGKIIFNPVVNNNTTLATGTNLGSATNP
750     LAEINFGSKGAANVDTVLNVGKGVNLYATNITTTDANVGSFIFNAGGTNI
800     VSGTVGGQQGNKFNTVALDNGTTVKFLGNATFNGNTTIAANSTLQIGGNY
850     TADFVASADGTGIVEFVNTGPITVTLNKQAAPVNALKQITVSGPGNVVIN
900     EIGNAGNYHGAVTDTIAFENSSLGAVVFLPRGIPFNDAGNRIPLTIKSTV
950     GNKTATGFDVPSVIVLGVDSVIADGQVIGDQNNIVGLGLGSDNDIIVNAT
1000    TLYAGIGTINNNQGTVTLSGGIPNTPGTVYGLGTGIGASKFKQVTFTTDY
1050    NNLGNIIATNATINDGVTVTTGGIAGIGFDGKITLGSVNGNGNVRFVDGI
1100    LSHSTSMIGTTKANNGTVTYLGNAFVGNIGDSDTPVASVRFTGSDGGAGL
1150    QGNIYSQVIDFGTYNLGISNSNVILGGGTTAINGKINLRTNTLTFASGTS
1200    TWGNNTSIETTLTLANGNIGNIVILEGAQVNATTTGTTTIKVQDNANANF
1250    SGTQTYTLIQGGARFNGTLGGPNFVVTGSNRFVNYGLIRAANQDYVITRT
1300    NNAENVVTNDIANSSFGGAPGVGQNVTTFVNATNTAAYNNLLLAKNSANS
1350    ANFVGAIVTDTSAAITNAQLDVAKDIQAQLGNRLGALRYLGTPETAEMAG
1400    PEAGAIPAAVAAGDEAVDNVAYGIWAKPFYTDAHQSKKGGLAGYKAKTTG
1450    VVIGLDTLANDNLMIGAAIGITKTDIKHQDYKKGDKTDVNGFSFSLYGAQ
1500    QLVKNFFAQGSAIFSLNQVKNKSQRYFFDANGNMSKQIAAGHYDNMTFGG
1550    NLTVGYDYNAMQGVLVTPMAGLSYLKSSDENYKETGTTVANKQVNSKFSD
1600    RTDLIVGAKVAGSTMNITDLAVYPEVHAFVVHKVTGRLSKTQSVLDGQVT
1650    PCISQPDRTAKTSYNLGLSASIRSDAKMEYGIGYDAQISSKYTAHQGTLK
1700    VRVNF
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Fig. 4. Overlapping rOmpB peptides observed from mass spectrometry. Sequence of the full length protein encoded by *R. conorii* rOmpB. The β-peptide region is underlined and tryptic peptides identified are in bold. This result is representative of more than 6 distinct identifications.

16M heat-resistant agglutinin 1 precursor BMEI0345 (PIR: AC3295; E-value = 0.45), and *Escherichia coli* strain 536 adhesin/virulence factor Hec (PRF: 2822341D). Despite the low level of homology by BLAST, multiple sequence alignment of these proteins clearly showed significant sequence homologies between these protein sequences along their entire lengths (Fig. 5). RC1281 exhibited marginal similarity (E-value = 0.003) with the opacity protein and related surface antigen domains (COG3637), and contained a signal peptide at its N-terminal (1–17 aa). However, RC1282 exhibited high similarity scores to COG3637 and the OmpA-like transmembrane domain (PFAM: PF01389). These domains (COG3637 and PF01389) are known to form a β-barrel domain integrated into the outer membrane and exhibiting a variety of functions.

4. Discussion

In the present study, we have identified two putative rickettsial adhesins probably involved in host cell binding. The outer membrane (and its components) is the initial site of interaction between rickettsiae and their host cells and thus is a key actor for entry and infection. The intracellular nature of these bacteria, associated with the absence of genetic tools, has hampered detailed study of the molecular mechanisms responsible for their pathogenicity [49]. Here we used a proteomic approach in conjunction with traditional overlay assays to identify possible rickettsial adhesins. Binding experiments performed on crude lysates of either *R. prowazekii* or *R. conorii* identified 2 proteins of 32–30 kDa which are specifically bound by eukaryotic cell lysates. Based on the membrane-impermeant properties of the biotin reagent, and as described in other published reports [8], we assume that most eukaryotic biotinylated proteins were cell surface proteins. The protein nature of rickettsial ligands was inferred from the observation that incubation of bacteria with trypsin suppressed binding in overlay assays. This observation fits well with experiments described by Li and Walker [25], demonstrating that binding of rickettsiae to mouse fibroblast cells was reduced by treatment of bacteria with trypsin or heat. Based on the fact that the rickettsial genome possesses several proteins exhibiting an MW of 32–30 kDa, direct identification of the rickettsial ligands after SDS–PAGE separation was problematic. We thus performed 2D-PAGE for better resolution. These experiments were performed using *R. conorii* and *R. prowazekii* membrane-enriched fractions and optimized conditions described in a previous work [38]. Identification of proteins by MALDI-TOF MS, which only provides peptide mass fingerprints and not sequence information, requires an efficient rickettsial purification procedure since contaminating eukaryotic proteins would result in undesirable background. In addition, good solubilization of protein samples is critical for high performance of 2D-PAGE, particularly for membrane-enriched extracts [34]. Results obtained using pH

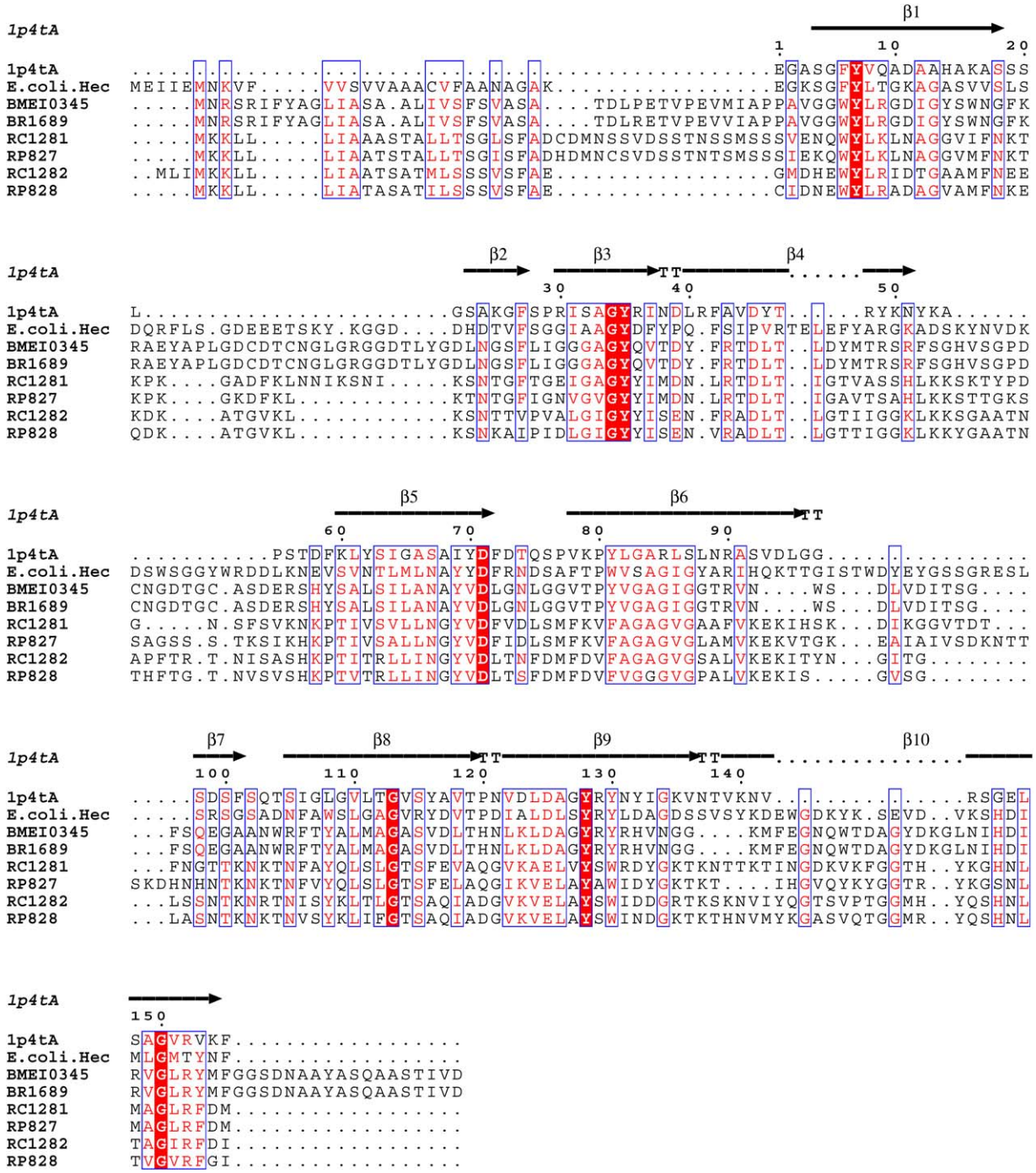

Fig. 5. Multiple sequence alignment of RC1281 and its homologues. Included sequences are from *R. conorii* (RC1281, RC1282), *R. prowazekii* (RP827, RP828), *Brucella suis* (BR1689), *Brucella melitensis* (BMEI0345), *E. coli* (Hec) and *Neisseria* (1p4tA). 1p4tA represents the sequence of neisserial surface protein A (PDB: 1P4T) with its secondary structures (β-strand and T representing turns) shown at the top of each alignment block. The sequence numbering refers to 1p4tA. Conserved residues were highlighted by colored letters and boxes.

6–11 strips yielded highly resolutive patterns allowing localization and identification of the rickettsial proteins that interact with biotinylated proteins. This analysis revealed that not only two proteins, but two trains of proteins, were recognized by eukaryotic cells. These putative adhesins were identified as the well known rickettsial membrane antigen rOmpB (otherwise termed Sca5) and a protein of unknown function encoded by ORFs RC1281 and RP828 in *R. conorii* and *R. prowazekii*, respectively. For the majority of assigned proteins, we noted a good correlation between experimentally observed and theoretical p*I* and MW values, except for the train of 32 kDa spots resolved between p*I* 8.5–9.5 and identified as rOmpB isoforms. The protein encoded by *rompB*/*sca5* is 168 kDa with a p*I* around 5.5. Further analysis of corresponding spectral peptides revealed that all peptides matched with the C-terminal end of rOmpB, termed the β-peptide, which is cleaved from full-length rOmpB [17]. Bioinformatic analysis rOmpB revealed that this protein is related to a large family of rickettsial cell

surface antigens and is the sole member of this family found within all sequenced rickettsial genomes. The β-peptide corresponds to the most conserved sequence region of this protein. These features are consistent with its a potential role as a genus-specific rickettsial adhesin and make it a potential target for a vaccine against all the bacteria of the genus.

The second rickettsial ligand recognized by biotinylated cells is a protein of unknown function encoded by RC1281 in *R. conorii* and RP828 in *R. prowazekii*. As illustrated in Fig. 5, RC1281, RC1282, RP827 and RP828 share striking homologies. Like the β-peptide, these proteins are ubiquitously present within the *Rickettsia* genus. Given the presence of a signal peptide in RC1281, and its significant sequence homology with membrane proteins as identified in multiple sequence alignment, RC1281 very likely forms a β-barrel structure within the rickettsial outer membrane, a location consistent with its function as a putative adhesin.

Previous studies have identified specific rickettsial proteins as adhesins, particularly rOmpA and rOmpB [26]. Although 2D-PAGE is a powerful tool for the separation and quantification of proteins, it has limitations in resolving proteins with high p*I*, hydrophobic characteristics, and high molecular weights [34]. Previous analysis of the *R. conorii* proteome through 2D-PAGE [38] indicates that rOmpA and rOmpB belong to this category and are not resolved in our gels. However, while both abundant proteins are present in SDS–PAGE separated crude rickettsial lysates, they are not recognized in overlay assays. It is likely that the binding sites of rOmpB are exclusively located within the proteolytically cleaved β-peptide region. Recently reported adhesion assays implicating rOmpB in adherence of rickettsiae to Vero cells were performed using *E. coli* expressing the precursor protein [44]. The portion of the recombinant rOmpB that was involved was not determined. In fact, to our knowledge the size of *rompB*-encoded putative adhesin was never determined, with previous investigations being largely based on inhibitory effect of antibodies. Therefore, and this is also the case for rOmpA, it is clear that these in vitro studies may have excluded certain interactions that occur in vivo and that require particular conditions of pH and salt concentration.

In this post-genomic period, we have taken advantage of genome sequences of rickettsiae and the development of proteomic tools to identify 2 putative rickettsial adhesins. Additional studies are necessary to confirm the adhesin function of these proteins. Further characterization of these putative rickettsial adhesins, their receptors, as well as other molecules that interact with host components, will increase our understanding of the pathogenesis of rickettsiae. Further advances in the field of genetic transformation of these microorganisms could lead to significant improvements in identification of rickettsial virulence factors.

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