Self-association of the Shigella flexneri IcsA autotransporter protein

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INTRODUCTION

Shigella spp. are highly adapted human pathogens that cause bacillary dysentery and extensive global morbidity and mortality (Levine et al., 2007). Ingested shigellae invade colonic epithelial cells where they multiply within the host cell cytoplasm and become motile via the polymerization of host actin in a process termed actin-based motility (ABM) (Suzuki et al., 1996, 1998, 2002). Motile bacteria are able to infect adjacent cells, enabling the lateral spread of the focus of infection throughout the epithelium. Actin polymerization is initiated by the shigella IcsA (VirG) protein that is distributed at the cell poles, (Bernardini et al., 1989; Lett et al., 1989) and it recruits the host neural Wiskott–Aldrich syndrome protein (N-WASP), a key regulator of the actin cytoskeleton (Goldberg, 2001; Snapper et al., 2001; Suzuki et al., 1998). N-WASP and other members of the Wiskott–Aldrich syndrome protein (WASP) family function as a link between signalling pathways and de novo actin polymerization by recruiting the actin-polymerizing complex Arp2/3, initiating actin-polymerization-driven host cell motility and morphological changes (Miki & Takenawa, 2003; Yarar et al., 1999). IcsA is essential for ABM and thus Shigella flexneri virulence (Kotloff et al., 1996, 2002; Lett et al., 1989; Makino et al., 1986; Sansonetti et al., 1991).

IcsA is a member of the autotransporter (AT) family of proteins, which is the largest family of extracellular proteins in Gram-negative bacteria (Pallen et al., 2003). A prototypical AT protein consists of: an N-terminal signal sequence that facilitates export across the inner membrane, an internal passenger domain that exerts the effector function and a C-terminal translocation domain required to direct export of the protein across the outer membrane (OM) via the Bam complex and to form a β-barrel OM anchor for the extracellular passenger domain (Henderson et al., 2004; Jain & Goldberg, 2007; Peterson et al., 2010).

A distinct subfamily of ATs has been shown to be trimeric and IcsA is related to a subgroup of self-associating ATs (SAATs) that mediate bacterial aggregation and biofilm formation (Cotter et al., 2005; Klemm et al., 2006; Meng et al., 2011). Emerging evidence suggests that a subset of conventional ATs are capable of oligomerization in the OM. In these proteins, oligomerization is facilitated through interactions either between adjacent effector domains (Gangwer et al., 2007; Swanson et al., 2009; Xicohtencatl-Cortes et al., 2010) or translocation domains.
(Marin et al., 2010; Müller et al., 2005; Veiga et al., 2002). How broadly applicable oligomerization might be within the large AT family of proteins remains to be determined. Indeed, some ATs have specifically been shown to exist as monomers (Hritonenko et al., 2006; Marin et al., 2010), and crystal structures of translocation domains obtained from EspP and NaIP suggest a monomeric existence (Barnard et al., 2007; Oomen et al., 2004). The biological significance of AT oligomerization remains unclear. IcsA and demonstrated the functional relevance of IcsA self-plaque formation, and hence intercellular spreading, OM and detected the existence of direct IcsA–IcsA monomers (Hritonenko et al., 2008). The large AT family of proteins remains to be determined.

The aim of our study was to investigate the state of IcsA in the OM environment, which is fundamental to understanding the interaction of IcsA with host proteins, such as N-WASP, and to S. flexneri virulence. In this study, we have shown that IcsA is present within a complex in the OM and detected the existence of direct IcsA–IcsA interactions within this complex. The identification of negative–dominant IcsA mutants that influenced S. flexneri plaque formation, and hence intercellular spreading, provided genetic evidence of direct IcsA–IcsA interaction and demonstrated the functional relevance of IcsA self-association.

METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Growth media and growth conditions. S. flexneri strains were grown from a Congo Red-positive colony as previously described (Morona et al., 2003). All bacterial strains were routinely cultured in Luria–Bertani medium. Bacteria were grown in medium with antibiotics for 16 h with aeration, then subcultured 1:50 and grown to mid-exponential phase by incubation with aeration for 2 h at 37 °C. Where appropriate, the medium was supplemented with ampicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), kanamycin (50 µg ml⁻¹) or tetracycline (50 µg ml⁻¹). Mueller–Hinton broth and agar were used to culture strains in the presence of trimethoprim (10 µg ml⁻¹).

DNA methods. Escherichia coli K-12 strain DH5α was used for routine cloning and general cloning techniques, and PCR and DNA sequencing were performed as described previously (May & Morona, 2008).

Chemical cross-linking. Cross-linking with dithio-bis(succinimidyldimpropionate) (DSP; Pierce) was performed as previously described by Thanabalu et al. (1998). Mid-exponential-phase cultures were washed in buffer (120 mM NaCl, 20 mM sodium phosphate; pH 7.2) and DSP was added to each sample at a final concentration of 0.2 mM. Samples were incubated for 30 min at 37 °C. Cross-linking was then quenched with 20 mM Tris pH 7.5, samples were washed in buffer and resuspended in SDS-PAGE sample buffer (Lugtenberg et al., 1975) either with or without β-mercaptoethanol. Samples were then heated to 60 °C for 5 min, prior to being resolved by SDS-PAGE. Cross-linking of cells from strains RMA2205, RMA2208 and RMA2209 was performed as above, except that after quenching the cells were lysed by passage through a French pressure cell, the lysate was centrifuged at 100,000 g for 1 h and the pelleted whole membranes resuspended in SDS-PAGE sample buffer either with or without β-mercaptoethanol. Formaldehyde cross-linking was performed as previously described by Prossnitz et al. (1988). Mid-exponential-phase bacteria were washed in 10 mM K₂HPO₄/ KH₂PO₄ buffer and resuspended, formaldehyde was added at a final concentration of 0.5% and then they were incubated for 1 h at room temperature. Samples were then washed once again as above and resuspended in SDS-PAGE sample buffer. Aliquots of each sample were heated at either 60 °C for 10 min (preserving cross-links) or 100 °C for 20 min (breaking cross-links) prior to SDS-PAGE.

Construction of plasmids. pKMRM252 was constructed by subcloning the EcoRI–SalI fragment of pIcsA encoding IcsAWT into likewise-digested pBBR1MCS-2. FLAG- and BIO-tagged derivatives of IcsA were constructed as follows. Complementary oligonucleotides (Table 2) encoding either the FLAG epitope (IcsA.FLAG-F1, IcsA.FLAG_R1; DYKDIDDKD) or the BIO sequence (KM1.BIO_F, KM1.BIO_R; GLNDHEFAQKIEWHI) were annealed as described by Enninga et al. (2005). The resultant dsDNA possessed NotI-compatible 5’ overhangs and was ligated to the unique NotI site within the linker-insertion of pKMRM1, producing plasmids pKMRM250 (encoding icsA87.FLAG) and pMG55 (encoding icsA87.BIO). To enable co-expression with pMG55, the EcoRI–SalI fragment of pKMRM250 encoding icsA87.FLAG was subcloned between the EcoRI and SalI sites of the compatible plasmid pBBR1MCS-2, producing plasmid pKRM270. We confirmed that IcsA87.FLAG and IcsA87.BIO were functionally comparable to IcsAWT by introducing either pKRM250 or pMG55 into RMA2041 (Table 1) and performing plaque assays (data not shown).

Reciprocal co-purification of FLAG- and BIO-tagged IcsA. E. coli UT5600 was transformed with pKRM270 and pMG55, enabling co-expression of IcsA87.FLAG and IcsA87.BIO, resulting in strain MG157. Control strains were also generated that expressed untagged IcsA87 [pKRM1M] with either IcsA.FLAG [pKRM270] (MG250) or IcsA.BIO [pMG55] (MG251). Strains MG157, MG250 and MG251 additionally carried pCY216 (Chapman-Smith et al., 1994).

Cultures (5 l) of MG157, MG250 and MG251 were grown for 16 h at 30 °C and extraction of outer-membrane proteins from each strain was performed at 4 °C as described by Veiga et al. (2002). Briefly, bacteria were pelleted, resuspended in TN buffer (20 mM Tris/HCl pH 8.0, 10 mM NaCl), lysed by passage in a French pressure cell at 12 000 p.s.i. (≈ 82.7 MPa) and centrifuged at 100 000 g for 1 h. The pellet was solubilized in TN buffer supplemented with 1.5% (v/v) Triton X-100 (Sigma) for 30 min and centrifuged at 100 000 g for 1 h. The resulting pellet was solubilized in TN buffer supplemented with 1% (w/v) Zwittergent 3-14 (Calbiochem) for 30 min and centrifuged at 100 000 g for 1 h. The supernatant containing solubilized outer-membrane proteins was collected and diluted to 0.1% (w/v) Zwittergent 3-14 with TN buffer. Solubilized material was then used in affinity purification using FLAG M2 resin (Sigma) or Dynabeads MyOne Streptavidin T1 (Invitrogen). Samples and beads or resin were incubated overnight at 4 °C, washed six times for 1.5 h in 8 ml TN buffer containing 0.1% (w/v) Zwittergent 3-14. Protein was eluted in SDS-PAGE sample buffer from FLAG M2 resin; streptavidin–Dynabeads were heated at 100 °C for 5 min to release bound protein. Protein was then pelleted at 16 000 g for 5 min and the supernatant then diluted in SDS-PAGE sample buffer for electrophoresis.

SDS-PAGE and Western blotting. Samples were separated on 7.5 or 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h in TTBS (Tris-buffered saline, 0.05% Tween-20) containing 5% skimmed milk and incubated with either rabbit anti-IcsA polyclonal antibody (Van Den Bosch et al., 1997), streptavidin–horseradish peroxidase (HRP) (Chemicon) or with rabbit anti-FLAG M2 (Sigma) in TTBS overnight. After three 10 min washes in TTBS the membrane was incubated with HRP-conjugated, goat anti-rabbit antibodies or HRP-conjugated, goat anti-mouse secondary antibodies (Biomediq DPC).
Table 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain/plasmid</th>
<th>Relevant characteristics*</th>
<th>Reference/source</th>
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<td>DH5α</td>
<td><strong>endA hisD supE44 thi-1 recA1 gyrA relAΔ (lacZYA-argF) U169</strong> [φ80 dlac (lacZ) M15] phoA</td>
<td>Gibco-BRL</td>
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<td>UT5600</td>
<td>F’ ara-14 leuB6 secA6 lacY1 proC14 txy-67 Δ(ompT-fepC)266 entA403 trpE88 rfrD1 rpsL109 ysl-5 met-1 thi-1</td>
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<td>MG157</td>
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<td>This study</td>
</tr>
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<td>MG250</td>
<td>UT5600 [pKMRM270, pKMRM1, pCY216]</td>
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<tr>
<td>MG251</td>
<td>UT5600 [pKMRM252, pMG55, pCY216]</td>
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<td><strong>S. flexneri</strong></td>
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<td>RMA2041</td>
<td>2457T ΔicsAΔ; TeR</td>
<td>Van Den Bosch &amp; Morona (2003)</td>
</tr>
<tr>
<td>RMA2107</td>
<td>RMA2041 ΔmolDΔ; KmR [pIcsA]</td>
<td>Van Den Bosch &amp; Morona (2003)</td>
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<td>RMA2041 [pD10-1]</td>
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<td>RMA2041 [pD10-1 virG3]</td>
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<td>KMRM277</td>
<td>RMA2041 [pKMRM34] [pKMRM270]</td>
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<td><strong>Plasmids</strong></td>
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<td>pBBR1MCS-2</td>
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<td>pCY216</td>
<td>Encodes BirA; CmR</td>
<td>Chapman-Smith et al. (1994)</td>
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<td>pD10-1</td>
<td>Encodes VirG (same as IcsAWT);TpR</td>
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<td>pD10-1 virG3</td>
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<td>Suzuki et al. (1996)</td>
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<td>pD10-1 virG4</td>
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<td>Suzuki et al. (1996)</td>
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<td>May &amp; Morona (2008)</td>
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<td>pMG55</td>
<td>pBR322 encoding IcsA&lt;sub&gt;187&lt;/sub&gt;–BIO; ApR</td>
<td>This study</td>
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*ApR, ampicillin-resistant; CmR, chloramphenicol-resistant; KmR, kanamycin-resistant; TeR, tetracycline-resistant; TpR, trimethoprim-resistant.

Table 2. Oligonucleotides used in this study

<table>
<thead>
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<th>Oligonucleotides</th>
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<tr>
<td>KM1_BIO_F</td>
<td>GGCCCTGAAACGACATCTTTGAAAGTCAGAAAATCGAATGGCAC</td>
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<tr>
<td>KM1_BIO_R</td>
<td>GCCCTGACCATGATTGGATTCAAGAAGACTGAACAGCAAC</td>
</tr>
<tr>
<td>IcsA_FLAG_F1</td>
<td>GGCGCGACTACAAAGGACGATGACGACAAG</td>
</tr>
<tr>
<td>IcsA_FLAG_R1</td>
<td>GCCCTTGTGCTCTAGGCTCGGTAGACTG</td>
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</table>

*Bold nucleotides comprise the NotI overhang of the annealed epitopes.
for 2 h, washed three times in TTBS and then three times in Tris-buffered saline. The membrane was incubated with Chemiluminescence Substrate (Sigma) for 1 min. Chemiluminescence was detected by exposure of the membrane to X-ray film (AGFA) and the film was developed using a Curix 60 automatic X-ray film processor (AGFA).

**Plaque assays.** Plaque assays were performed with HeLa cells as described previously (May & Morona, 2008). Briefly, HeLa cells were maintained in minimum essential medium (MEM), containing 10% FCS with penicillin and streptomycin, and grown to confluence overnight in six-well trays. HeLa cells were washed twice with Dulbecco’s PBS (D-PBS) and once in Dulbecco’s modified Eagle’s medium (DMEM) prior to inoculation with mid-exponential-phase bacteria diluted in DMEM. At 90 min post-infection the inoculum was aspirated and an overlay [DMEM, 5% FCS, 20 μg gentamicin ml⁻¹, 0.5% agarose, 0.1% Neutral Red solution (Gibco-BRL)] was added at either 24 h or 48 h post-infection and plaque formation observed 6–8 h later. Plaques were, in general, visible without staining at 48 h.

**Indirect immunofluorescence of whole bacteria.** Indirect immunofluorescence labelling of bacteria was performed as described previously (May & Morona, 2008). Briefly, equivalent numbers of mid-exponential-phase bacteria were fixed in formalin [3.7% (v/v) paraformaldehyde in 0.85% saline] and either centrifuged onto poly-L-lysine-coated coverslips for microscopic analysis or kept in suspension for flow cytometric analysis. Bacteria were incubated with the desired primary antibody, which was diluted 1 : 100 in PBS with 10% FCS with penicillin and streptomycin, and grown to confluence in six-well trays. HeLa cells were washed twice with D-PBS and incubated with medium containing gentamicin for a further 1.5 h. The monolayer was washed, formalin-fixed and permeabilized with 0.1% Triton X-100. After blocking in 10% FCS the infected cells were incubated with polyclonal anti-Shigella LPS-O antigen (Oag) (Denka Seiken Co.) to label bacteria. After subsequent washing, cells were incubated with Alexa 594-conjugated donkey anti-rabbit antibodies (Molecular Probes). F-actin was visualized by staining with FITC–phalloidin (0.1 μg ml⁻¹, Sigma).

**RESULTS**

**In situ chemical cross-linking of IcsA**

In order to determine if IcsA is present within a protein complex, *in situ* chemical cross-linking was performed. Mid-exponential-phase cultures of *S. flexneri* ΔicsA [pIcsA] (RMA2090, Table 1) were treated with the membrane-permeable, cross-linking agent DSP, and whole-cell lysates were subjected to SDS-PAGE and Western blotting with an anti-IcsA antibody. We reproducibly detected the presence of a high molecular mass (HMM) complex following cross-linking (Fig. 1, lane 2). This complex had an apparent molecular mass greater than 460 kDa, as determined by comparison with HighMark protein standard (Invitrogen). Formation of this HMM product was reversed by the addition of β-mercaptoethanol, which can cleave the disulphide bond in the spacer arm of DSP to allow separation of the cross-linked products (Fig. 1, lane 1). This HMM complex could also be detected following cross-linking with formaldehyde (Fig. S1, available with the online version of this paper).

Oligomerization of the *Neisseria gonorrhoeae* IgA protease and *E. coli* AIDA has been suggested to be mediated through interaction of individual translocation domains (Müller et al., 2005; Veiga et al., 2002). We examined previously characterized IcsA effector domain deletion mutants (Suzuki et al., 1996) for formation of HMM complexes. DSP cross-linking of IcsA<sub>Δ508–730</sub> (RMA2208) or IcsA<sub>Δ103–507</sub> (RMA2209) produced HMM products comparable to IcsA<sub>WT</sub> (RMA2205) (Fig. 1, lanes 3–6). These data indicated that no individual region within IcsA<sub>Δ103–730</sub> is alone responsible for formation of the HMM complex; we cannot exclude that multiple interactions within this region are involved. Alternatively, the data indicate that IcsA HMM complex formation may be mediated by a region outside of the studied deletions, namely by the translocation domain (IcsA<sub>Δ758–1102</sub>).
LPS is a major constituent of the OM of Gram-negative bacteria, consisting of three major parts – the lipid A, the core polysaccharide and the Oag polysaccharide chain. The Oag component of LPS has been shown to influence both IcsA function and surface localization, restricting the protein to the cell pole (Hong & Payne, 1997; Morona & Van Den Bosch, 2003a, b; Sandlin et al., 1995). Rough LPS (R-LPS), which lacks the Oag component, was reported to allow IcsA diffusion away from the cell pole (Robbins et al., 2001). We hypothesized that cross-linking of IcsA into a HMM product may be dependent on the spatial confinement of IcsA proteins to the pole or be facilitated by lateral LPS–IcsA interactions. Hence, we examined the IcsA complex formation in the absence of LPS–Oag. HMM IcsA-related complexes were observed following DSP cross-linkage of IcsA expressed in the R-LPS strain S. flexneri DicsA DrmlD [pIcsA] (RMA2107), indicating that complex formation occurred independent of LPS–Oag (Fig. S1).

Co-immunoprecipitation of epitope-tagged IcsA proteins

Chemical cross-linking had indicated the presence of IcsA within a HMM complex and we were particularly interested in determining if IcsA is able to self-associate. We applied reciprocal co-purification of differentially epitope-tagged IcsA proteins to determine whether IcsA–IcsA interactions were taking place within this putative complex.

Sites permissive for epitope insertion within the IcsA passenger domain had been previously identified (May & Morona, 2008). The IcsAi87 protein has a five amino acid linker-insertion at amino acid 87 and when expressed in S. flexneri, was found to be comparable to wild-type (IcsA WT) with respect to: (i) levels of production, (ii) polar localization at the bacterial surface and (iii) function, determined by assaying plaque formation on HeLa cell monolayers as a measure of intercellular spreading ability (May & Morona, 2008). Consequently, IcsAi87 was chosen for epitope tagging by exploiting a unique NotI restriction site within the linker. Either a synthetic FLAG epitope (DYKDDDDK) or BIO epitope (GLNDIFEAQKIEWH, a substrate for metabolic biotinylation by the BirA biotin-protein ligase) (Cull & Schatz, 2000) was introduced into IcsAi87, as described in Methods.

E. coli UT5600 strains expressing both epitope-tagged IcsA proteins together (IcsAi87::FLAG and IcsAi87::BIO; MG157, Table 1) or tagged proteins individually with control IcsAWT were generated (MG250 and MG251, Table 1). Overexpression of BirA increased the levels of biotinylated IcsAi87::BIO, while retaining the high specificity of the biotinylation reaction (data not shown). Hence, all co-expression strains additionally carried plasmid pCY216 that expressed the BirA enzyme (Chapman-Smith et al., 1994). Neither the insertion of the FLAG nor the BIO epitope affected the function of IcsAi87 (data not shown).

The OM fractions of the co-expression strains were isolated and purification strategies directed towards each epitope were performed independently; FLAG M2 resin was used for purification of IcsAi87::FLAG and streptavidin–Dynabeads were used for purification of biotinyl–IcsAi87::BIO. Co-purification of tagged IcsA proteins was assessed by Western blotting using either an antibody conjugate (FLAG epitope) or streptavidin conjugates (biotin-modified BIO epitope) in each of the eluents. Following purification of IcsAi87::FLAG with FLAG M2 resin from OMs of strain MG157, biotinyl–IcsAi87::BIO was found to co-purify with it (Fig. 2a, lane 2). Taking into consideration that any IcsAi87::BIO that remained unbiotinylated could not be detected with streptavidin, our data suggest that the amount of co-purified biotinyl–IcsAi87::BIO we observed was significant. Under the same conditions, when OMs of strain MG251 that expressed IcsAWT were used, biotinyl–IcsAi87::BIO was not detected in the eluted sample (Fig. 2a, lane 1), showing that the FLAG M2 resin was specific for the FLAG epitope. Similarly, when biotinyl–IcsAi87::BIO was purified from OMs of strain MG157 using streptavidin–Dynabeads, IcsAi87::FLAG was found to co-purify with it.

![Fig. 2. Co-purification analysis. E. coli K-12 strains (MG157, MG250 and MG251) were grown, then Zwittergent 3-14-solubilized OM fractions were prepared and purified through FLAG-agarose resin (a) or streptavidin–Dynabeads (b), as described in Methods. Samples were subjected to SDS-PAGE and Western blotting with either a rabbit anti-FLAG M2 antibody or streptavidin–HRP conjugate, as indicated.](image-url)
(Fig. 2b, lane 2). However, under the same conditions, IcsA<sub>87</sub>:FLAG did not purify from OMs of strain MG250 that co-expressed the untagged control protein IcsA<sub>WT</sub> (Fig. 2b, lane 3), showing that the streptavidin–Dynabeads were specific for the biotin-modified BIO epitope. Based on these results we conclude that the IcsA self-association occurs in the OM and this self-association is resistant to disruption by the detergent Zwittergent 3-14.

**Identification of negative-dominant IcsA<sub>i</sub> mutants**

Having detected the existence of IcsA–IcsA interactions by co-purification, we investigated if IcsA self-association impacted on its function in intracellular motility. We hypothesized the existence of IcsA mutations that would exert negative dominance on IcsA function in ABM and intercellular spread when co-expressed with IcsA<sub>WT</sub>. A collection of IcsA<sub>i</sub> insertion mutants has been previously identified (May & Morona, 2008) and some of these were screened for negative dominance in intercellular spreading when co-expressed with IcsA<sub>WT</sub> in a S. flexneri KMRM254 background (data not shown). Two mutants (IcsA<sub>563</sub> and IcsA<sub>677</sub>) exerted a clear negative-dominant phenotype when expressed with either IcsA<sub>WT</sub> (Fig. 3a) or the functionally equivalent IcsA<sub>87</sub>:FLAG (Fig. S2). Individually, IcsA<sub>563</sub> and IcsA<sub>677</sub> are unable to recruit N-WASP (Fig. S3) or efficiently generate F-actin tails and promote plaque formation on HeLa cell monolayers (May & Morona, 2008). When either mutant was co-expressed with IcsA<sub>WT</sub>, F-actin tails could still be detected by FITC–phalloidin staining of fixed monolayers infected with S. flexneri (Fig. 3b). However, efficient F-actin tail formation is required for efficient intercellular spreading. As a sensitive measure of the proficiency of the detected F-actin tails to drive intercellular spreading, the ability of the co-expression strains to form plaques on HeLa cell monolayers was assessed. Plaque formation could not be detected when IcsA<sub>563</sub> was co-expressed with IcsA<sub>WT</sub> and co-expression of IcsA<sub>677</sub> with IcsA<sub>WT</sub> resulted in only a few small plaques (<1 mm in diameter) that were markedly smaller than those formed by the control strain KMRM255 (which typically formed between 50 and 100 plaques per well, averaging 6.4 mm in diameter) (Fig. 3a). The defective plaque formation clearly indicated that the F-actin tails formed by these strains were not comparable to the wild-type, as they were not able to facilitate efficient ABM and intercellular spreading.

**Fig. 3.** Plaque and F-actin tail formation by complemented strains. (a) Relative size and frequency of plaque formation on HeLa cell monolayers by S. flexneri strains expressing either IcsA<sub>WT</sub> (RMA2090), IcsA<sub>563</sub> (KMRM111) or IcsA<sub>677</sub> (KMRM134) alone, and S. flexneri co-expressing IcsA<sub>WT</sub> with IcsA<sub>563</sub> (KMRM256), IcsA<sub>677</sub> (KMRM258) or IcsA<sub>WT</sub> control (KMRM255). Plaque assay was performed as described in detail in Methods. (b) Immunofluorescence microscopy of F-actin tail formation by intracellular S. flexneri strains expressing either IcsA<sub>WT</sub> (RMA2090), IcsA<sub>563</sub> (KMRM111) or IcsA<sub>677</sub> (KMRM134) alone, and S. flexneri co-expressing IcsA<sub>WT</sub> with IcsA<sub>563</sub> (KMRM256), IcsA<sub>677</sub> (KMRM258) or IcsA<sub>WT</sub> control (KMRM255). HeLa cells infected with S. flexneri were labelled with anti-LPS antibodies and Alexa 594-conjugated donkey anti-rabbit antibodies, and F-actin was labelled with FITC–phalloidin as described in detail in Methods. Strains were assessed in three independent experiments. Arrows indicate F-actin tails. Bar, 10 μm.
Negative dominance of IcsA\textsubscript{i} mutants on IcsA\textsubscript{WT} does not arise due to defects in production, export or polar localization of the wild-type protein

The observation of negative dominance when IcsA\textsubscript{WT} was expressed with IcsA\textsubscript{i} mutants could have arisen by titration by the mutant proteins of factors required for IcsA synthesis, polar localization and surface presentation, resulting in a decrease in function of IcsA\textsubscript{WT}. We sought to scrutinize these possibilities by using a tagged version of IcsA\textsubscript{WT} (IcsA\textsubscript{i87}::FLAG) in \textit{S. flexneri} ΔicsA strains additionally expressing IcsA\textsubscript{i563} or IcsA\textsubscript{i677} mutants. IcsA\textsubscript{i87}::FLAG retains a wild-type-equivalent function in plaque formation (Fig. S2). We confirmed that the IcsA\textsubscript{i563} and IcsA\textsubscript{i677} mutants had a negative-dominant effect on IcsA\textsubscript{i87}::FLAG with respect to plaque formation (Fig. S2).

The levels of IcsA\textsubscript{i87}::FLAG protein during co-expression with IcsA\textsubscript{i563} and IcsA\textsubscript{i677} were then assessed by Western blotting with anti-FLAG M2 antibodies. IcsA\textsubscript{i87}::FLAG production was equivalent when expressed alone from either pKMRM250 (pBR322 derivative) or pKMRM270 (pBBR1MCS-2 derivative), confirming the suitability of the plasmids for use in co-expression studies (data not shown). Production of IcsA\textsubscript{i87}::FLAG when co-expressed with IcsA\textsubscript{WT} in KMRM275 was comparable to production of IcsA\textsubscript{i87}::FLAG when expressed alone in KMRM273 (Table 1; Fig. 4a). Despite being unable to form detectable plaques, the strain co-expressing IcsA\textsubscript{i677} with IcsA\textsubscript{i87}::FLAG
exhibited comparable levels of FLAG-tagged functional IcsA; co-expression of IcsAi563 and IcsAi87::FLAG resulted in a slight decrease in FLAG-tagged IcsA (Fig. 4a). Additionally, we quantified the level of surface-exposed IcsAi87::FLAG by flow cytometry. Co-expression of IcsAi87::FLAG with either mutant did not result in a detectable reduction in the amount of functional protein at the bacterial surface (Fig. 4b). Moreover, the surface distribution of IcsAi87::FLAG, as detected by immunofluorescence, was polar when co-expressed with either IcsAWT, IcsAi563 or IcsAi677 (Fig. 4c).

Taken together, these data suggest that the negative-dominant phenotype conferred by IcsAi563 and IcsAi677 during co-expression with active IcsA results from a net functional defect at the cell surface, and was not due to these mutants affecting the expression, export or localization of functional IcsA proteins.

**DISCUSSION**

Subversion of the host cell actin regulatory network enables shigella infection to spread throughout the human intestinal epithelium. IcsA is both necessary and sufficient to potentiate ABM through activation of N-WASP. The nature of IcsA interaction with N-WASP remains poorly understood. In light of data demonstrating that the conventional AT, IgA protease from *N. gonorrhoeae*, forms an oligomer in the OM, we sought to characterize the properties of IcsA in the OM. In this study, we tested the hypothesis that IcsA is able to self-associate.

The self-association of IcsA was supported by the presence of HMM IcsA-related complexes that were detected following cross-linking of whole cells with DSP or formaldehyde. While IgA1 protease in *vitro* oligomerization was reported as homo-pentameric (Veiga *et al.*, 2002), our findings do not exclude the possibility that IcsA oligomerization is hetero-oligomeric. The reciprocal co-purification and genetic interaction data we have presented in this study strongly suggest the existence of IcsA–IcsA interactions within the HMM oligomer.

Support for the existence of direct IcsA–IcsA interactions within HMM complexes was provided by observation of negative-dominant genetic interactions of IcsA linker-insertion mutants (IcsAi563 and IcsAi677) on IcsA function in intercellular spreading (assessed with both IcsAWT and IcsAi87::FLAG). One of two explanations can account for negative-dominant phenotypes (Herskowitz, 1987). The first is titration of factors away from the functional IcsA protein by co-expression of a mutant IcsA protein. Since no defects were observed in functional IcsA whole-cell protein levels, in cell surface expression or in polar targeting, the factors that underpin the respective processes were not titratable by IcsA mutants. Similarly, the examined IcsA mutants cannot recruit N-WASP (May & Morona, 2008) and titration of this host cell ligand from functional IcsA was improbable. These findings indicated that titration could not explain the negative-dominant phenotype. Instead, we favour the alternative explanation of negative dominance: the products of the functional *icsA* and mutant *icsA* alleles functionally interact *in vivo*. We suggest that during co-expression, IcsAi mutants are included into mixed complexes with functional IcsA proteins. The inclusion of IcsAi proteins renders these complexes defective for efficient F-actin tail formation, as evidenced by defective intercellular spreading. This deficiency in the negative-dominant co-expression strains occurs despite levels of functional IcsA that, when expressed alone, can efficiently potentiate intercellular spreading. Clearly, IcsA–IcsA interactions are functionally important.

We have demonstrated for the first time, to our knowledge, biochemical and genetic evidence of IcsA self-association. Furthermore, the observed negative dominance of certain inactive IcsA mutants is consistent with the fact that self-association is important for the biological function of the protein. Although N-WASP and WASP family members are activated by an array of different molecules, these activators act by allosteric relief of N-WASP auto-inhibition (Kim *et al.*, 2000) and by facilitating clustering and multimerization of active N-WASP (Padrick *et al.*, 2008; Padrick & Rosen, 2010), often by their own oligomerization. Therefore, we hypothesize that the self-association of IcsA may play a role in N-WASP clustering. In our study the incorporation of defective IcsAi563 or IcsAi677 proteins into IcsAWT-containing complexes could interfere with efficient spatial clustering of activated N-WASP, thereby accounting for the reduced efficiency of intercellular spreading. In this way, IcsA would appear analogous to other bacterial and host proteins that either interact with or functionally mimic WASP family members. Most of these have been shown to either directly self-associate (enterohaemorrhagic *E. coli* intimin-tir), to spatially cluster (*Listeria monocytogenes* ActA) or they are inherently multivalent with internal repeats (enterohaemorrhagic *E. coli* EspF, EspFv and Tccp) (Alto *et al.*, 2007; Campellone *et al.*, 2008; Footer *et al.*, 2008; Sallee *et al.*, 2008; Touzé *et al.*, 2004). Significantly, our data demonstrate self-association of yet another protein known to activate a WASP family protein, which has emerged as an important feature in the regulation of the host actin cytoskeleton.

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